

THE CELLULAR DEGRADATION OF THE LOW DENSITY  
LIPOPROTEIN RECEPTOR AND ITS LIGAND.

By

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ABSTRACT

The cellular degradation of the low density lipoprotein (LDL) receptor, and its ligand, LDL, were investigated in order to clarify certain mechanistic aspects of these important processes.

Long-term lymphoblastoid cell lines and cultured human skin fibroblasts were used to examine the fate of  $^{125}\text{I}$ -LDL subsequent to its uptake via receptor-mediated endocytosis. In both cases, binding activity was saturable, depended on the presence of calcium ions in the medium, and was calculated to have an equilibrium dissociation constant at  $4^\circ\text{C}$  of  $2 \mu\text{g } ^{125}\text{I}\text{-LDL/ml}$ . No high-affinity binding was detected when the ligand was modified by acetylation. After incubating the monolayers at  $37^\circ\text{C}$ , LDL/LDL receptor complexes were internalized, and the receptors were recycled back to the surface within about 10 minutes. Apolipoprotein B in the LDL particles was largely degraded to the amino acid level: chloroquine, a lysosomotropic agent, inhibited the formation of the  $^{125}\text{I}$ -LDL degradation products. Cells obtained from a number of heterozygous and homozygous familial hypercholesterolemic patients, as expected, bound markedly reduced amounts of ligand. The half-life of  $^{125}\text{I}$ -LDL was measured after it had been introduced into cultured fibroblasts by one of the following processes: (i) uptake via receptor-mediated endocytosis in human skin fibroblasts with normal LDL receptors, or (ii) incorporation via scrape-loading into fibroblasts defective

in LDL receptor content. The half-lives obtained were about 1 hour and 50 hours, respectively, indicating that efficient degradation of LDL occurred only when it was delivered to lysosomes via receptor-mediated endocytosis.

A pulse-chase procedure using [<sup>35</sup>S]-methionine, and involving specific immunoisolation, was used for the accurate quantitation of radioactive LDL receptors. Using this protocol, the half-life of labeled LDL receptors on normal fibroblast monolayers was shown to be  $11.7 \pm 2.2$  hours (n=10), irrespective of the status of the cells with respect to prior up- or downregulation of the receptors. The presence of LDL or 25-hydroxycholesterol in the culture medium also did not affect the half-lives of the receptors. A similar half-life was obtained using an internalization-defective fibroblast cell line (GM 2408A), indicating that receptor clustering in coated pits and receptor recycling are not prerequisites for receptor degradation. LDL receptor turnover was not altered by the prior removal of terminal sialic acid residues from the receptors as well as other plasma membrane glycoproteins. Degradation was unaffected by the presence of leupeptin in the chase-incubation medium, and also occurred when the cells were incubated at 18°C, albeit at a slower rate than was the case at 37°C. The latter two results strongly indicate that the rate-limiting step(s) in receptor degradation do not involve the lysosomes. Cycloheximide, a protein synthesis inhibitor, slowed LDL receptor degradation to a marked degree, probably by stopping the synthesis of a short-lived protein essential to the degradation process. Monensin,

chloroquine or ammonium chloride all appeared to produce sequestration of receptors in intracellular sites, accompanied by a dramatic increase in the receptor degradation rate. In the case of monensin, the increased rate of degradation was partially inhibited by leupeptin. From this, it can be surmized that the rate-limiting initial step(s) in LDL receptor degradation are mediated by non-lysosomal proteases, while lysosomal enzymes possibly participate in the degradation of the receptors when the latter are trapped in endosomes.

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ABBREVIATIONS AND SYMBOLS.

AcLDL	= acetylated LDL
Apo B (or apo E)	= apolipoprotein B (or apolipoprotein E)
ASGP	= asialoglycoprotein
B <sub>max</sub>	= maximum binding capacity
BSA	= bovine serum albumin
°C	= degrees Celsius
cDNA	= complementary DNA
CHAPS	= 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate
CHO	= Chinese hamster ovary
cpm	= counts per minute
CURL	= compartment of uncoupling of receptor and ligand.
DMEM	= Dulbecco's modification of Eagle's Minimum Essential Medium.
DMEM/LPDS	= medium containing LPDS instead of fetal calf serum.
dpm	= disintegrations per minute
EDTA	= ethylenediamine tetracetic acid
EGF	= epidermal growth factor
EGTA	= ethyleneglycol-bis-(aminoethyl ether)-N,N,N',N'-tetracetic acid
EMEM	= MEM without L-methionine & L-glutamine & with 2 g/l sodium bicarbonate
ER	= endoplasmic reticulum
FCS	= fetal calf serum
FH	= Familial hypercholesterolemia
g	= acceleration due to gravity (all values given are for g <sub>av</sub> )
HDL	= high density lipoprotein
Hepes	= N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HIFCS	= heat-inactivated fetal calf serum
3HMG CoA reductase	= 3-hydroxy-3-methylglutaryl coenzyme A reductase
hr	= hour
HSF	= human skin fibroblasts
IDL	= intermediate density lipoprotein
IgG/A/M	= immunoglobulin G/A/M
kb	= kilobase
kd	= kilodalton
$K_D$	= equilibrium dissociation constant
LDL	= low density lipoprotein
LPDS	= lipoprotein-deficient serum
MEM	= Eagle's Minimal Essential Medium buffered with Earle's salts.
MEM/LPDS	= medium containing LPDS instead of FCS
mRNA	= messenger RNA
mwt	= molecular weight
PAGE	= polyacrylamide gel electrophoresis
PBS	= phosphate buffered saline
PBS-ALB	= PBS + 0.2% albumin
PMSF	= phenylmethylsulphonyl fluoride
rpm	= revolutions per minute
S.D.	= standard deviation $[\sum(X-x)^2/(n-1)]^{1/2}$
SDS	= sodium dodecyl sulphate
SDS-PAGE	= sodium dodecyl sulphate polyacrylamide gel electrophoresis
$t_{1/2}$	= half-life $[y_t = y_{inr} + (y_0 - y_{inr})e^{-kt}]$
TCA	= trichloroacetic acid
tris	= tris(hydroxymethyl) amino methane
VLDL	= very low density lipoprotein
WHHL rabbits	= Watanabe heritable hyperlipidemic rabbits.

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Many fundamental advances in biology have been inspired by analyses of human genetic diseases (19, 74, 110, 183, 238). One such classic example has involved the study of the disease known as Familial Hypercholesterolemia (FH), which was first described in 1938 by Carl Muller (166) as a disorder that produced high blood-cholesterol levels and myocardial infarctions in young people. About three decades later, Khachadurian (124) and Fredrickson and Levy (73) showed that FH could exist clinically as either the heterozygous or the homozygous form. The research team headed by M.S. Brown and J.L. Goldstein began an extensive in vitro biochemical analysis of this disease in the early 1970's; their studies have lead to the discovery of a cell surface receptor for a plasma cholesterol transport protein known as low density lipoprotein (LDL), and to the elucidation of a pathway, generally known as receptor-mediated endocytosis, via which this receptor mediates the feedback control of cholesterol synthesis.

Recently, findings by Dietschy have emphasised the central role played by hepatic LDL receptors in most mammals (reviewed in reference 59). Circulating levels of LDL-cholesterol are determined mainly by: (i) the rate of LDL production, and (ii) the rate of LDL uptake. Both of these events are mediated largely by the liver. Furthermore, most of the hepatic LDL uptake occurs via receptor-mediated processes. Alterations in dietary cholesterol input are usually adequately compensated for by altered hepatic (and intestinal) cholesterol synthesis rates, thereby ensuring the maintenance of constant circulating plasma cholesterol

levels. However, when the adaptive alterations in cholesterol synthesis are unable to compensate adequately for the changed cholesterol input, the level of LDL receptor activity in the liver may be varied as an additional means to restore the normal cholesterol balance.

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### 1.1 Receptor-mediated endocytosis.

Biological membranes per se are impermeable to many molecules. Various cellular processes exist which are able to circumvent this problem - they are termed "endocytosis", and may include the processes of "pinocytosis" (the ingestion of small portions of the extracellular environment) or "phagocytosis" (the uptake of large, particulate material) (6). The significant disadvantage of such processes is that molecules are internalized in proportion to their concentration in the extracellular environment. Viewed in this perspective, the value of receptor-mediated endocytosis (the process whereby cell surface proteins - "receptors" - are able to recognize, bind with high affinity and internalize specific groups of extracellular macromolecules), is obvious. More than 25 different receptors are known to participate in receptor-mediated endocytosis (Table 1.1).

There is one feature common to all receptor-mediated endocytotic pathways: receptors always move to coated pits (specialized regions of the surface membrane containing the protein, clathrin), which then invaginate rapidly into the cell during endocytosis to form endocytotic vesicles.

**Table 1.1: Some examples of receptors that participate in receptor-mediated endocytosis.**

Receptor specific for:	Ligand function	Found on	Route type*	Fate of Internalized ligand	Selected refs
LDL	cholesterol transport	HSF, SMC**, lymphocytes, endothelial cells	A	lysosomal degradation; cholesterol retained by cells	2 83 85 87
Trans-ferrin	iron transport	hepatocytes reticulocytes erythroblasts	B	iron retained by cells	99 115
Insulin	polypeptide hormone	hepatocytes lymphocytes adipocytes 3T3 cells	A	lysosomal degradation. Ligand also delivered to nuclei & golgi	156 241
EGF	protein growth factor	fibroblasts 3T3 cells	C	lysosomal degradation	37 91 156
ASGP*	non-transport plasma protein	hepatocytes	A	lysosomal degradation	9 242
macro-globulin	serum protease inhibitor	fibroblasts macrophages 3T3 cells	A	lysosomal degradation	157 251
Maternal IgG	immune complexes	fetal yolk sac, neonatal intestinal cells	D	transferred intact to basal surface of cells where IgG is discharged	146 194 197

\* = the pathway followed subsequent to receptor-mediated endocytosis, as detailed in section 1.1.

\*\* = smooth muscle cells

# = asialoglycoproteins

Adapted from Reference 82.

Movement of receptors into coated pits is not always a spontaneous event: although many receptors enter coated pits continuously, even in the absence of ligand eg. the receptors for: LDL (1, 15), insulin (135) and asialoglycoproteins (24, 254), there are some, eg. the epidermal growth factor (EGF) receptor (66, 205), which remain diffusely distributed on the cell surface, and only become trapped in coated pits once bound to ligand.

There is substantial evidence to indicate that many endocytotic receptors can enter cells in the same coated pits, and may be delivered to the same acidified endosomes (182, 252). Thereafter, the pathways diverge, and the receptor-ligand complexes may follow one of four different routes (as discussed below, and illustrated in Fig. 1.1).

**Route A: Receptor is recycled, ligand is degraded.**

This pathway was described originally for the LDL receptor in 1974 (83, 84), and has subsequently been shown to be the classic route whereby many diverse ligands are endocytosed (Table 1.1). Upon exposure to the low pH of the endosome, an organelle alternately known as "CURL" (the compartment of uncoupling of receptor and ligand), the receptor-ligand complexes dissociate (30, 98). The ligand is transported to lysosomes, where it is subsequently degraded, whereas the receptors are thought to leave the endosome in recycling vesicles originating as tubular endosomal extensions in which receptors gather. These extensions probably pinch off from the main endosome body, recycle to the cell surface, and fuse with the plasma membrane (76). Aulinskas *et al*

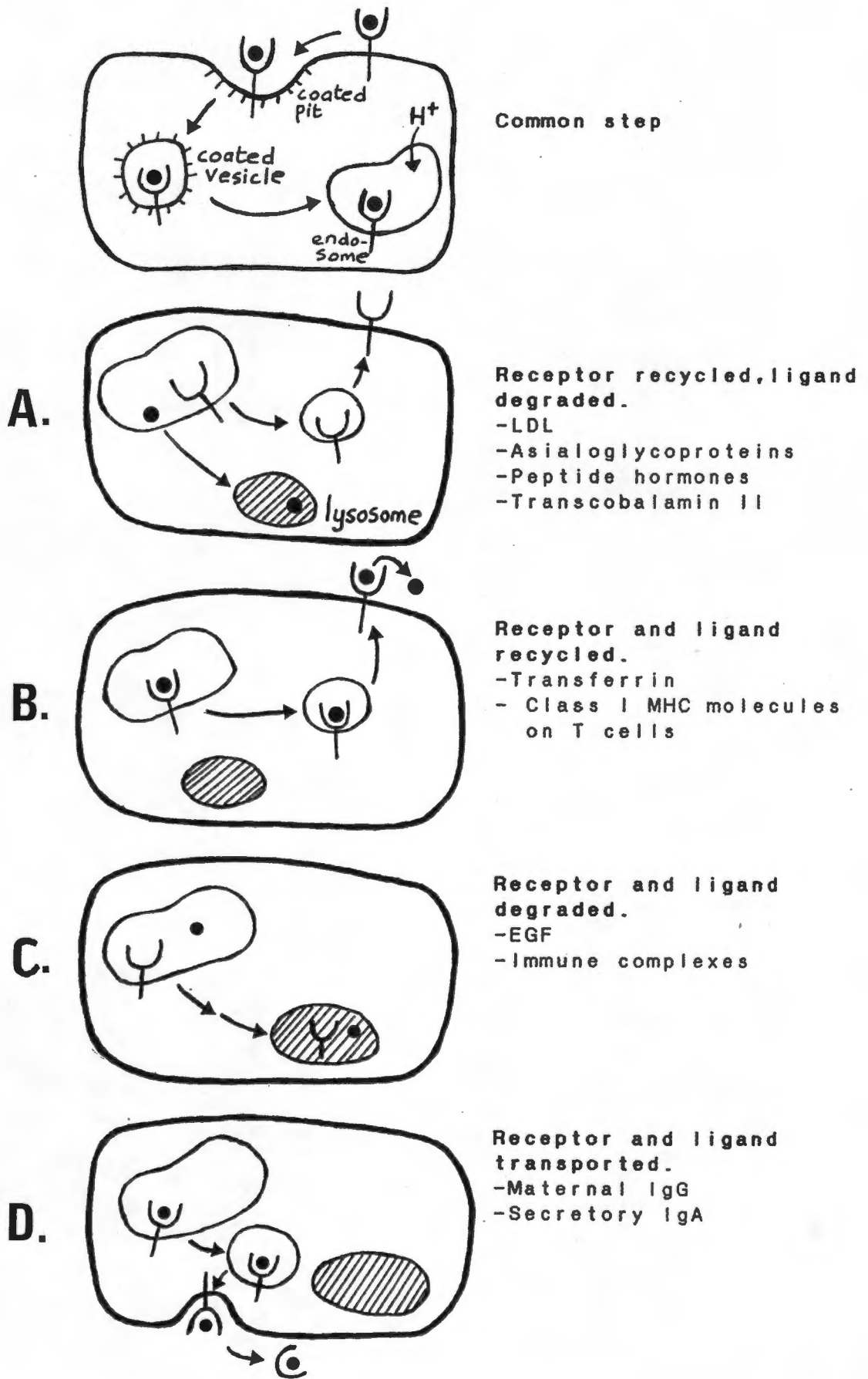



Fig. 1.1: Four pathways of receptor-mediated endocytosis.

Code:  = receptor

● = ligand

Adapted from Reference 88.

(12) have proposed the existence of an additional pathway operational in the LDL receptor system: in studies performed using cultured smooth muscle cells and cultured human skin fibroblasts (HSF), these investigators showed that about half the LDL entering the cells via coated vesicles in the steady state was subsequently discharged from the cytoplasm by a route they termed "retro-endocytosis"; the remainder of the endocytosed ligand was degraded lysosomally.

In route A, receptors may be re-used once every 10 to 20 minutes (the time taken to traverse one endocytotic cycle), which means that a single receptor can mediate the uptake of many ligands during its lifespan. If one assumes an average receptor half-life of 30 hours, and a recycling time of 15 minutes, then such a receptor should (in theory) be able to recycle 120 times. Recycling necessitates a receptor structure which is adequately stable to withstand repeated passage through the acidic endosomal environment without denaturation. Conformational changes sufficient to enable the release of ligands, but not such that receptors are reversibly denatured, must occur within the endosomes.

**Route B: Receptor and ligand are recycled.**

This pathway was originally described for the transferrin receptor (169), and is characterized by the fact that the

receptor-ligand complex does not dissociate in the endosome. Apotransferrin [a major serum glycoprotein which transports iron from sites of absorption (intestine) and storage (liver) to tissue cells (48)], binds iron very tightly to form ferrotransferrin, which is the ligand for the cell surface transferrin receptor. At the pH 5 of the endosomes, the iron dissociates from ferrotransferrin, but the apotransferrin remains bound to the receptor, and is recycled to the cell surface. Subsequent exposure to the neutral pH of the extracellular interstitial fluid causes the dissociation of receptor and its iron-free ligand (48). Recently, work by other investigators has suggested that this type of recycling pathway may provide the mechanism whereby cells of the immune system process and present antigen to effector cells (for review, see 184).

**Route C: Receptor and ligand are degraded.**

This pathway, characterized originally for the EGF receptor, involves the degradation of both components of the receptor-ligand complex, probably because of co-transport to the lysosome. It is likely that dissociation of the complex is an endosomal event, since EGF is known to dissociate from its receptor at low pH (60). The majority of the EGF receptors do not recycle to the cell surface, but are targeted to the lysosomes and degraded there. This mechanism provides for an effective ligand-induced downregulation of receptors.

**Route D: Receptor and ligand are transported.**

This routing has been described in greatest detail for the receptor that carries polymeric immunoglobulin A (IgA) and immunoglobulin M (IgM) across epithelial surfaces, eg. across liver cells for excretion into the bile (222).

The operation of four known pathways for the routing of receptor and ligand subsequent to uptake via receptor-mediated endocytosis is indicative of the existence of multiple mechanisms for the intracellular sorting of receptors. Furthermore, within each pathway, there occurs the continuous and precise movement of the membrane-embedded receptor proteins from one organelle to the next, as well as the segregation from neighbouring membrane proteins that do not follow the same route, at each stage. Accuracy in such sorting and targeting events is essential; to this end, each receptor probably comprises multiple functional domains, and regions which facilitate the sequential interaction with other macromolecules, to enable successive transport to various intracellular sites. In those cases where receptors are normally recycled, there must exist some additional mechanism(s) capable of partitioning the receptors into a recycling route normally, and a degradation pathway eventually.

The physiologically important LDL receptors, which bind cholesterol-carrying LDL particles, are recycling receptors, whose structure has been well elucidated (see section 1.2.4).

## 1.2 The LDL receptor.

### 1.2.1 LDL receptor purification and cDNA cloning - a historical perspective.

Until recently, the LDL receptor had been studied extensively in intact cells and isolated membranes by biochemical, genetic and ultrastructural techniques (2, 82 and 85), but it had not been purified. In 1979, Schneider *et al* (208) solubilized the LDL receptor from bovine adrenal cortex membranes using the nonionic detergent octylglucoside (the bovine adrenal cortex is an organ that contains a relative abundance of LDL receptors - about  $1 \times 10^5$  molecules per cell - see Section 2, Table 2.3 for comparative receptor numbers in two other cell types). Using a solid-phase assay, they were able to show that the solubilized receptors exhibited normal  $^{125}\text{I}$ -LDL binding characteristics (208). The following year, these investigators partially purified the LDL receptor protein using, sequentially, DEAE-cellulose chromatography, agarose gel filtration and phosphatidylcholine-acetone precipitation (211), to achieve a 350-fold purification relative to the activity of the starting material. This preparation was injected into rabbits, and a polyclonal antibody to the LDL receptor was isolated (21). Studies with these antibodies showed that there was immunologic cross-reactivity of LDL receptors from bovine adrenal cortex, human fibroblasts, canine liver and adrenal gland; these results provided the first indication that the

LDL receptor structure had been widely conserved among animal species and tissues (21). In 1982, Schneider et al described the procedure for the complete purification of the LDL receptor from bovine adrenal cortex membranes (209) - this entailed the procedures described above, followed by affinity chromatography on LDL coupled to Sepharose 4B, and elution of the receptor with suramin (an inhibitor of LDL/LDL receptor interactions). The purified receptor retained all of the binding properties of the LDL receptor of intact cells and crude membranes. Monoclonal antibodies against the LDL receptor (designated "IgG-C7") were prepared as described (23), and were used to follow the LDL receptor processing (see section 1.2.2).

In 1983, Russell et al (199) used the polyclonal antibodies to the LDL receptor to enrich for the rare LDL receptor messenger RNA (mRNA) by polysome immune purification. A complementary DNA (cDNA) library was constructed from the purified mRNA; this was screened with two families of oligonucleotides derived from the amino acid sequence of a cyanogen bromide fragment of the bovine protein. Ultimately, these methods enabled the isolation of a partial cDNA for the bovine LDL receptor (199). This was then used as a probe to isolate a fragment of the human LDL receptor gene, and, in turn, an exon probe from this genomic fragment was employed to isolate a cDNA clone which represented the complete 5.3 kilobase (kb) human LDL receptor mRNA (260). Yamamoto et al (260) transfected simian COS cells with the human LDL receptor cDNA linked to the SV 40 early promoter, and were able to demonstrate the expression of functional

cell surface receptors. These investigators used the oligonucleotide sequence of this cDNA to derive the complete amino acid sequence of the human LDL receptor (260).

These tools - viz. antibodies directed against the LDL receptor, and the receptor cDNA - were used to examine the synthesis, processing and structure of this important protein.

#### 1.2.2 LDL receptor synthesis and processing.

The LDL receptor is synthesised in the rough endoplasmic reticulum (ER) as a precursor (243) containing high-mannose N-linked carbohydrate chains and the core sugar of the O-linked chains (46). Addition of the O-linked sugars must occur either in the ER, or in a transitional zone between the ER and the golgi apparatus, because these sugars are added before the mannose residues of the N-linked chains are trimmed (a golgi event). The immature receptor, when subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), migrates as a single band corresponding to an apparent molecular weight (mwt) of 120 kilodalton (kd) (243). Tolleshaug et al (243), using a monoclonal antibody directed against the LDL receptor to immunoprecipitate biosynthetically radiolabeled receptors, have shown that within 30 minutes after synthesis, the apparent mwt of the receptors increases from 120 to 160 kd. This change coincides with the elongation of each of the 18 O-linked chains (by the addition of one galactose and one or

two sialic acid residues), and the conversion of the two asparagine-linked (N-linked) high-mannose oligosaccharide chains to the complex endoglycosidase H-resistant form (46). The apparent 40 kd increase in mwt is mainly due to a change in conformation of the protein as a result of the elongation of the clustered O-linked sugars (46, 49).

Approximately 45 minutes after synthesis, LDL receptors appear on the cell surface, where they gather in coated pits; binding of either of the two ligand types for which they are specific may occur (section 1.2.3). Irrespective of whether or not ligand binding has occurred, the coated pits invaginate to form coated endocytic vesicles within 3-5 minutes of their formation, and the process of receptor-mediated endocytosis takes place as described in section 1.1 (route A).

### 1.2.3 Nature of the ligand(s) for the LDL receptor.

It is postulated that the external, amino-terminal portion of each LDL receptor can potentially bind either (i) 4 apolipoprotein B (apo B) molecules located on separate LDL particles, with an equilibrium dissociation constant ( $K_D$ ) of about  $3 \times 10^{-9}$  M (77), or (ii) 4 molecules of apolipoprotein E (apo E), all probably located on a single lipoprotein particle, with a  $K_D$  of approximately  $10^{-10}$  M (149). Many investigators refer to the LDL receptor as the "apo B-100,E" receptor, for reasons which are obvious from (i) and (ii). (In all the experimental work reported in this thesis, LDL

was used as the source of ligand for the LDL receptor, hence the term "ligand" used with respect to the LDL receptor, refers in every case to the apo B containing LDL particles).

Apo E is an arginine-rich 33 kd protein, which may be associated with a variety of particles including, mainly, chylomicrons, very low density lipoproteins (VLDL) and certain species of high density lipoproteins (HDL). Hui et al (109) have defined the limited amino acid sequence responsible for apo E binding, partly on the basis of human mutations affecting binding to the LDL receptor. This part of apo E contains clustered positive charges, which is indicative of the important role of electrostatic interactions in the strength and specificity of this ligand-receptor interaction (the putative LDL receptor binding domain contains clustered negative charges; section 1.2.4).

Apo B is a protein which is known to exist as one of at least two isoforms; these are commonly known as apo B-100 and apo B-48. Apo B-100 is the only isoform present in LDL, and is the natural ligand for the LDL receptor (apart from apo E); apo B-48 does not bind to the LDL receptor. For the past 20 years, less than 5% of the apo B-100 sequence was known, despite the efforts of many investigators working in this field. Recently, however, two independent research teams have published the sequence of apo B-100 - one of the largest monomeric proteins yet known. Knott et al (129) used overlapping cDNAs, isolated from liver and Hep G2 cDNA libraries, to determine the complete 4536 amino acid sequence of the human apo B-100 precursor (mwt 514 kd).

Numerous lipid-binding structures were found to be distributed throughout the length of apo B-100, and are probably essential to the functioning of this protein in lipoprotein assembly and plasma lipoprotein integrity. There are two basic amino acid sequences (residues 3147-3157 and 3359-3367); the second of these bears some similarity to the receptor binding domain of apo E (residues 142-150) - the consensus being Arg-X-X-Arg-Lys-Arg-X-X-Arg/Lys. Yang *et al* (263) deduced the complete primary amino acid sequence of human apo B-100 by sequence analysis of apo B-100 tryptic peptides and overlapping partial cDNA clones. They mapped the non-random distribution of trypsin-accessible and inaccessible peptides, and on the basis of their results, were able to divide apo B-100 into 5 hypothetical domains in terms of the clustering of trypsin-accessible peptides. These investigators also defined a putative LDL receptor binding domain of this ligand by chemically synthesising the region corresponding to residues 3345-3381 of their sequence; the synthetic peptides could mediate binding and uptake by the LDL receptor. This sequence corresponds, approximately, with the second of the two basic amino acid sequences reported by Knott *et al* (129).

#### 1.2.4 The five structural domains of the LDL receptor.

An examination of the structure of the LDL receptor (Fig. 1.2), is of value since it yields some insight into the possible signals which direct the highly selective movement of receptors from one organelle to another.

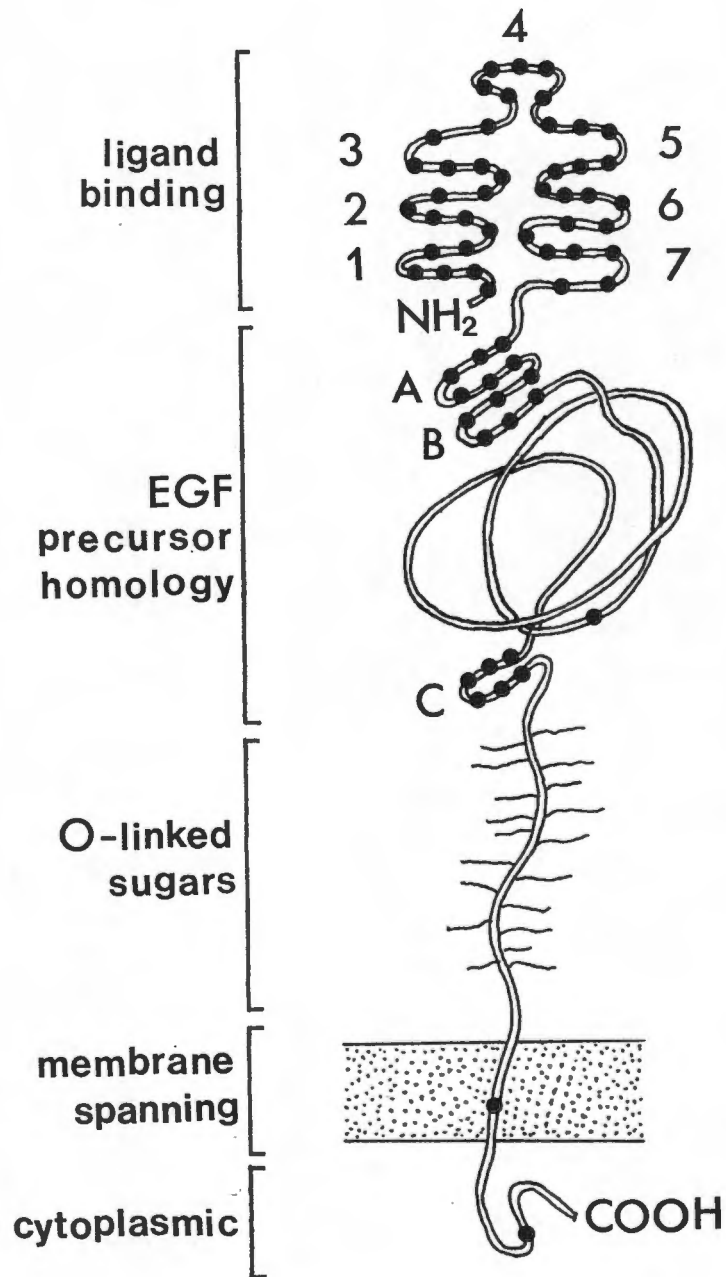


Fig. 1.2: Structure of the normal human LDL receptor.

Each dot denotes the position of one cysteine residue. Numbers represent the seven amino-terminal ligand binding domains.

Adapted from Reference 144.

There is a hydrophobic sequence of 21 amino acids at the extreme NH<sub>2</sub>-terminus of the LDL receptor; this is cleaved off immediately after translation, and functions as a classic signal domain to direct the receptor-synthesising ribosomes to the ER membrane. The mature receptor (minus the signal domain) consists of 839 amino acids (260).

#### Domain 1: Ligand binding.

This domain comprises the NH<sub>2</sub>-terminal 292 amino acids, and consists of a sequence of 40 amino acids that is repeated seven times, with some variation. Studies performed on intact cells with anti-peptide antibodies revealed that this domain is located on the external surface of the plasma membrane (212). Each of the seven repeat sequences contains six cysteine residues, which are in register for all of the repeats. It is likely that all of these cysteines are disulphide-bonded, since the receptor cannot be labeled with [<sup>3</sup>H]-iodoacetamide without prior reduction (88). This region of the receptor must therefore exist in a tightly cross-linked, convoluted state, which accounts for the extreme stability of the receptor-binding domain - the receptor can be boiled in strong denaturants yet still retain its binding activity, provided the disulphide bonds remain intact (88). A noticeable feature of each cysteine-rich repeat sequence, is a cluster of negatively charged amino acids near the COOH-terminus of each (228). The charges on these sequences are complementary to a cluster of positively charged residues (representing the binding site to the LDL receptor), in apo E (section 1.2.3 and references

111 & 149). Goldstein et al (88), have speculated that the negatively charged clusters of amino acids within the cysteine-rich repeat sequences of the LDL receptor constitute multiple binding sites, each capable of binding a single apo E molecule by attaching to its positively charged alpha-helix.

#### Domain 2: Homology with the EGF precursor.

The second domain of the LDL receptor consists of about 400 amino acids, and is approximately 35% homologous to a portion of the extracellular domain of the EGF precursor (198, 230, 260). There are three cysteine-rich amino acid repeat sequences in this domain (represented as A, B, and C in Fig.1.3, pg 34). To gain some insight as to the function of the second LDL receptor domain, Davis et al (50) have recently used oligonucleotide directed mutagenesis to delete the sequences encoding the 400 amino acids that comprise the EGF precursor homology region. The deletion-bearing plasmid was transfected into a line of mutagenized Chinese hamster ovary (CHO) cells that did not produce LDL receptors, and the cells which subsequently expressed high levels of the transfected receptor cDNA were used for analyses. Results obtained by these investigators demonstrated at least three functions for the EGF precursor homology region in the LDL receptor: (i) it is necessary for the binding of LDL, but not of beta-VLDL, to intact cells; (ii) it is needed for acid-dependent dissociation of beta-VLDL and (iii) its presence is needed for the receptor to participate in repeated recycling without degradation. The deleted LDL receptor performed many of its functions normally - such as

the processing and maturation of O-linked sugars - despite the removal of the entire precursor region (representing about half the protein). Furthermore, the protein was obviously not denatured, since it remained able to reach the cell surface and bind to  $^{125}\text{I}$ -labeled beta-VLDL, with normal kinetics.

### Domain 3: O-linked sugars.

The third domain of the LDL receptor lies immediately external to the membrane-spanning domain; it consists of a 58 amino acid sequence, 18 of which are either serine or threonine residues (228, 260). The entire domain is encoded by a single exon, and proteolysis studies have revealed that this region contains clustered carbohydrate chains attached by O-linkage (46, 198); each O-linked sugar chain consists of a core N-acetylgalactosamine, a single galactose and one or two sialic acid residues. Davis *et al* (49) deleted this domain from the cDNA for the human LDL receptor, and transfected the deleted cDNA into receptor-deficient hamster fibroblasts. Using this system, they were able to demonstrate conclusively that (i) the serine- and threonine-rich region of the LDL receptor is the site for addition of clustered O-linked carbohydrates (ii) the receptor contains a small number of isolated chains of O-linked carbohydrates in addition to the clustered chains; these are located in the  $\text{NH}_2$ -terminal portion of the protein (iii) the clustered O-linked carbohydrates are not essential for normal LDL receptor function in cultured hamster fibroblasts (49). To date, the precise function of this domain remains speculative - perhaps the clustered O-linked sugars merely

serve as struts to keep the receptors extended from the membrane surface so that they can bind to their ligands more efficiently (88).

**Domain 4: Membrane spanning region.**

This domain consists of a stretch of 22 hydrophobic amino acids; proteolysis experiments have confirmed that this region spans the plasma membrane (198, 260). Comparison of the amino acid sequences of the bovine and human LDL receptors has shown that this domain is relatively poorly conserved (88); 7 of the 22 amino acids in this region differ between human and cow, but all of the substitutions are also hydrophobic in character.

**Domain 5: Cytoplasmic tail.**

The fifth domain consists of a COOH-terminal segment of 50 amino acids which projects into the cytoplasm (198, 260). This sequence is strongly conserved among species: only 4 of the 50 amino acids differ between human and cow, and each of these substitutions is conservative with respect to the amino acid charge (88). The cytoplasmic domain of the LDL receptor plays an important role in mediating the clustering of the receptor into coated pits by interacting with clathrin itself, or with some protein associated with clathrin on the cytoplasmic side of the membrane (30, 82). Evidence for this is obtained from the molecular analysis of mutations at the LDL receptor locus which produce receptors capable of binding LDL normally, but which do not cluster in coated pits, and are not internalized. These mutations,

which also all produce defects in the cytoplasmic tail, are discussed in section 1.3.2.

#### 1.2.5 A comparison with other receptors.

The structure and transmembrane orientation of the LDL receptor is compared with that of five other well characterized coated pit receptors in Table 1.2. Comparison of the amino acid sequences has yielded no obviously conserved feature(s).

#### 1.2.6 The LDL receptor at a genetic level.

##### mRNA structure.

The human LDL receptor mRNA appears as a single species of approximately 5.3 kb on Northern blots (reviewed in reference 88), 2.5 kb of which comprises an unusually long 3' untranslated region. A remarkable feature of this untranslated region is the presence of  $2\frac{1}{2}$  RNA copies, within about 750 nucleotides, of a middle repetitive Alu sequence; these repeats generally occur once in every 5 000 bp in the human genome (section 1.3.3 and reference 206).

##### Gene structure.

The human LDL receptor gene, located on chromosome 19, spans more than 45 kb, and comprises 18 exons which show a striking correlation to the functional domains of the

**Table 1.2: A comparison of the structure of the LDL receptor with 5 other receptors that enter coated pits.**

Each protein spans the plasma membrane once.

Parameter	Receptors for....					
	LDL	Insulin	Trans-ferrin	ASGP	EGF	IgA IgM
No. of amino acids in mature receptor	839	1343	760	283	1186	755
No. of polypeptide chains	1	2	2	1	1	1
NH <sub>2</sub> terminal end is..	E*	E	I**	I	E	E
No. of amino acids in cytoplasmic portion	50	402	61	38	542	105
No. of potential sites for attachment of N-linked oligosaccharide chains.	5	34	6	3	12	2
Presence of clustered O-linked sugars	yes	no	no	no	no	no
Cysteine-rich regions	yes	yes	no	no	yes	no
Tyrosine kinase activity	no	yes	no	no	yes	no

\* = extracellular      \*\* = intracellular

Adapted from reference 88.

protein (Fig. 1.3). Other noticeable features of this gene include: (i) a multiple duplication of a single exon to produce a binding domain containing seven repeats of a single 40-amino acid sequence (section 1.2.4, and references 34 & 88 for comprehensive reviews), and (ii) a large 3' untranslated region of 2.5 kb (encoded for by exon 18), which includes several copies of a repetitive sequence of the Alu family.

### 1.3 Naturally occurring genetic defects in the LDL receptor.

Many naturally occurring mutations in the LDL receptor gene are known to exist; such mutations disrupt receptor functioning in various ways. Individuals who inherit one mutant LDL receptor gene (heterozygous for FH), produce about 50% of the number of normal receptors, so that in vivo, plasma LDL levels are doubled. Ultimately, the elevated plasma LDL levels lead to heart attacks by about 40 years of age in the afflicted persons. In individuals with two mutant LDL receptor genes (FH homozygotes), few or no functional LDL receptors are produced by the cells. This results in plasma LDL levels 8 to 10 times higher than is normal; the patients develop atherosclerosis at a very early age, and usually die from heart attacks in childhood. Many of the apparent FH homozygotes are, in fact, compound heterozygotes, who have inherited different mutant alleles from each parent. The research team headed by M.S. Brown and J.L. Goldstein (University of Dallas, Texas, U.S.A.),

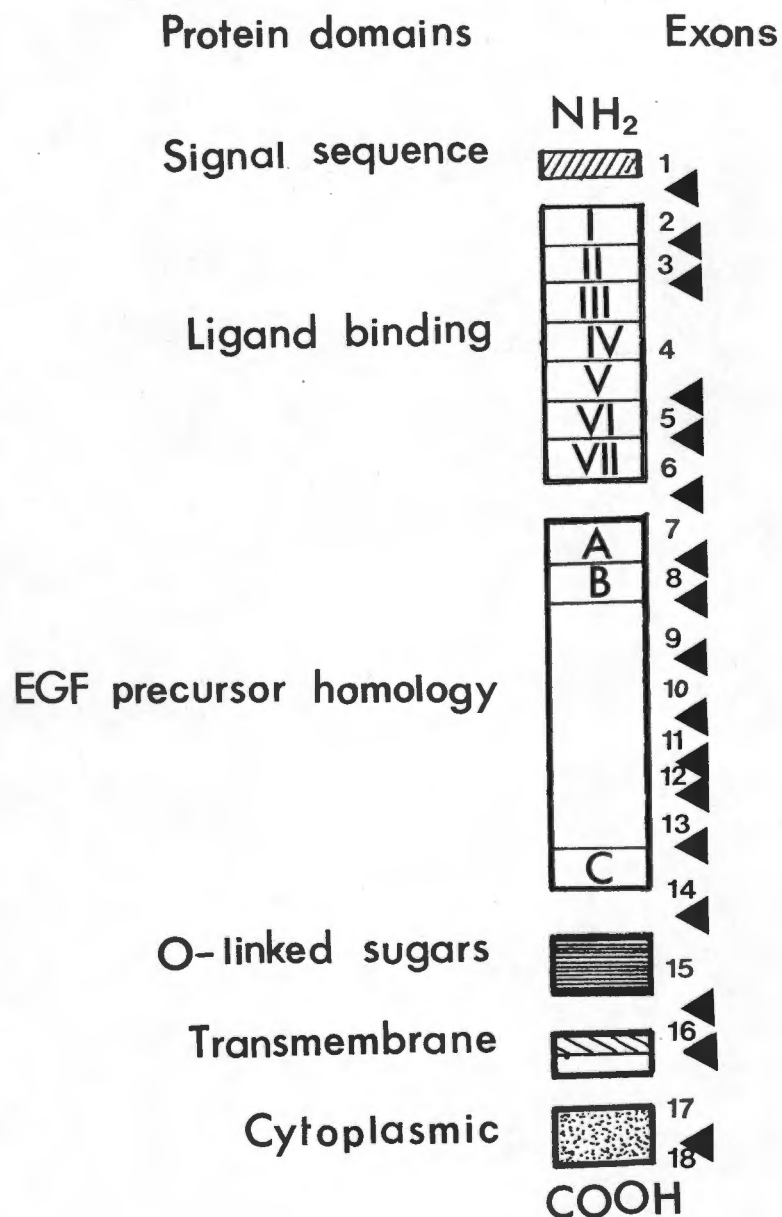


Fig. 1.3: The normal human LDL receptor - a correlation between exon organization and protein domains.

Arrow heads indicate intron positions. Boxes indicate exons. The 7 cysteine-rich amino acid repeats in the LDL receptor binding domain are numbered I to VII. The 3 cysteine-rich repeats in the domain that is homologous with the EGF precursor, are lettered A, B and C.

Adapted from Reference 228.

has studied cultured skin fibroblasts obtained from over 100 FH homozygotes; their results have revealed that the mutations can be divided into four broad classes, on the basis of their effects on receptor structure and function (Table 1.3, and see reference 88 for a comprehensive review).

The analysis of these mutations - especially at the molecular genetic level - has provided valuable insight into the structure-function relationships of the LDL receptor, and, on a more general plane, has served as a model system for the elucidation of the receptor-mediated endocytosis pathway. The recent investigations carried out on the LDL receptor mutants are reviewed below, and summarized in Table 1.4. This review is up-to-date at the time of writing (June, 1987). It should be noted, however, that this area is the subject of intense research at present. With each new mutant that is investigated and reported, further fascinating insight into this field is gained.

#### 1.3.1 Population groups with increased incidence of FH.

The prevalence of heterozygous FH appears to be approximately 1 in 500 among the general population of most countries, while that of the homozygous form of this disease is about 1 in 1 million. However, two population groups are known to exist in which the frequency of the homozygous condition is more than 10-fold higher, viz. the Lebanese population (124) and the white Afrikaans-speaking South

Table 1.3: Mutations at the LDL receptor locus that produce FH.

Class of mutation	Precursor processing	Mwt (kd)*		LDL binding to intact cells	Frequency in FH patients
		Prec*	Mature		
1	No precursor detectable	none	none	none	common
2	Precursor not processed	100	100	none	rare (Lebanese)
		120	120	none	common
		135	135	none	rare
2 variant	Slow precursor processing	120	160	reduced	rare (WHHL rabbits** & Afrikaners)
3	Normal processing	100	140	reduced	rare
		120	160	reduced	common
		170	210	reduced	rare
4	Normal processing	110	150 )	normal binding,	rare
		120	160 )	defective	rare
		115	155 )	internalization	rare

\* = apparent molecular weight (in kd) on SDS-PAGE.

# = Precursor

\*\* = Watanabe heritable hyperlipidemic rabbits (see section 1.3.1).

Adapted from reference 88.

Table 1.4: Summary of some naturally occurring and constructed mutations in the LDL receptor gene.

Mutation yields insight into...	Designation	Genetic Cause	Structural Consequence	Receptor Characteristics
movement of receptors to cell surface	FH 563	7-12 amino acid deletion in exon 4	deletion in 5 <sup>th</sup> repeat of domain 1	"slow processing" of 120 kd to 160 kd.
	WHHL rabbit	4 amino acid deletion	occurs in 3 <sup>rd</sup> repeat of domain 1	slow processing (as above)
	FH 264	nonsense mutation at amino acid 660: TGC to TGA	receptor has only domains 1 & 2	95 kd not processed. Slow exit from ER
Domain 1	FH 626	exon 5 deleted	6 <sup>th</sup> repeat of ligand binding domain missing	100 kd to 140 kd. Binds VLDL but not LDL.
	FH 295	duplication of introns 1 to 8	duplication of exons 1-8	170 kd to 210 kd. Low LDL binding
Domain 2	con-structed	deletion of nucleotides 293-692	400 amino acid deletion (domain 2 is removed)	72 kd to 125 kd. VLDL (but not LDL) binds
Domain 3	con-structed	deletion of 144 base pairs in exon 15	48 amino acids encoded by exon 15 are removed	100 kd to 120 kd. Normal binding

Mutation yields insight into...	Designation	Genetic Cause	Structural Consequence	Receptor Characteristics
Domain 4	FH 781	deletion of 7.8 kb. Intron 15 joined to exon 18	normal domains 4 & 5 missing. 55 new amino acids at COOH terminus	155 kd mature. >90% secreted
	FH 274	deletion of 5 kb	domains 4 & 5 missing	110 kd to 150 kd. Mostly secreted.
Domain 5	FH 380	single base substitution at aa* 807: TAT to TGT	intact receptor	120 to 160 kd No clustering in pits
	FH 763	4 base duplication in region of aa 796	domain 5 has 6 normal, & 8 additional, aa	115 to 155 kd No clustering in pits
	FH 683	single base substitution at aa 792: TGG to TGA	only 2 aa in domain 5	115 kd to 155 kd
constructed		24 different mutations in domain 5		
	FH 381	5 kb deletion Intron 15 joined to exon 13		null phenotype
	T.D.	exons 13 & 14 deleted	Truncated domain 2 & no domains 3, 4 & 5	biosynthetic study not yet done

\* = amino acid

African population (43, 248). In both cases, this is attributable to a high prevalence of heterozygosity, coupled with a high incidence of consanguinity, which increases the proportion of homozygotes. The first clear delineation of the existence of homozygous FH was done by Khachadurian (124) on studies of the Lebanese population.

Lehrman et al (144) have recently described a mutant LDL receptor gene (the "Lebanese allele", FH 264) that produces a shortened receptor protein lacking three domains: the region of clustered O-linked carbohydrates, the membrane-spanning region, and the cytoplasmic tail. Using genomic cloning techniques, these investigators were able to establish that the defect was caused by a single nucleotide substitution that creates a premature termination codon at amino acid 660, thus eliminating 180 residues from the mature protein. The resultant truncated protein retains only two domains - a complete ligand-binding region, and a partial EGF precursor homology region. After synthesis, most of the receptors remained within the cell for several hours in the unprocessed form, which possibly indicates that the shortened receptors leave the ER at an abnormally slow rate, most likely due to the abnormal folding of the truncated protein.

Biosynthetic studies have shown that the LDL receptor gene mutation existing in the South African population produces a 120 kd receptor precursor that is processed abnormally slowly to the mature 160 kd form (210). To date, the molecular basis of this novel mutation has not been

reported. It is of interest to note that there exists a similar "slow processing" LDL receptor mutation in a strain of rabbits designated Watanabe hereditary hyperlipidemic (WHHL) rabbits (210). Yamamoto et al (259) have cloned and sequenced cDNAs from these and normal rabbits, and have shown that this defect arises from an in-frame deletion of 12 nucleotides that eliminates four amino acids from the cysteine-rich ligand binding domain of the LDL receptor. They also examined one slow-processing patient (FH 563, not one of the Afrikaner patients), and found a similar small deletion in exon 4 of the LDL receptor gene. Such mutations do not remove any of the cysteine residues, but probably are sufficient to alter the folding pattern of the cysteine-rich region, thereby preventing the formation of proper disulphide bonds. It is feasible that such mutant proteins may be trapped by detection mechanisms ("gatekeeper proteins") that are specifically designed to prevent the movement of abnormally folded proteins to the cell surface. Such mechanisms might be of crucial biological importance since the surface appearance and/or secretion of even a few denatured molecules may produce harmful immune responses.

### 1.3.2 Internalization-defective mutations.

The importance of coated pits in receptor-mediated endocytosis was originally revealed by the study of these mutations at the cellular level. In 1985, Lehrman et al (145) characterized a mutation in the structural gene for a patient - designated FH 274 - who possessed a (maternally

inherited) internalization-defective mutation for the LDL receptor (and a paternal null allele). A comparison of the nucleotide sequences of the normal and FH 274 genes showed the presence of a 5 kb deletion, which eliminated the exons encoding the membrane-spanning and the carboxyl terminal cytoplasmic receptor domains. Intrastrand recombination between two repetitive sequences of the Alu family (section 1.3.3), appeared to be the cause of the deletion. The truncated receptors lacked the membrane-spanning and cytoplasmic regions, and were largely secreted into the culture medium. The small fraction of the receptors remaining adherant to the cell surface did bind LDL, but were unable to cluster in coated pits. These studies represented the first elucidation of an LDL receptor mutation at a molecular level, and the results, among other things, were indicative of a putative role of clathrin/clathrin-associated cytoplasmic proteins in receptor internalization. The splicing pattern of the deleted mRNA, and the precise COOH terminus of the protein have not yet been identified. [Three other LDL receptor mutations have recently been described, all of which involve intra-strand recombinations between the Alu sequences; these are detailed in 1.3.3 (a)].

The same research group subsequently isolated the segment of the LDL receptor gene encoding the COOH-terminal cytoplasmic domain from the cells of two other individuals with the same internalization-defective phenotype (140). Their findings showed that one mutant gene (from patient FH 683), contained a single base substitution which changed a tryptophan codon

(TGG) to a termination codon (TGA); this produced a receptor with only 2 amino acids in the cytoplasmic domain. The other mutant gene, from patient FH 763, was found to contain a four-base duplication, which produced a frameshift that altered the reading frame; six of the normal amino acids and eight additional amino acids were found in the cytoplasmic tail of the LDL receptor encoded by this gene. Both of the mutations described above produced LDL internalization-defective receptors, with truncated cytoplasmic domains, but unaffected membrane spanning regions. These findings supported the hypothesis that the cytoplasmic domain is required for the clustering of LDL receptors.

In 1986, these investigators reported on the nature of the LDL receptor mutation existing in patient J.D. (FH 380) (the internalization-defective phenotype was first documented in fibroblasts from this individual) (51). DNA sequence analysis revealed the substitution of a tyrosine codon for a cysteine codon at residue 807 in the cytoplasmic domain of the receptor. These researchers used oligonucleotide-directed mutagenesis to produce the J.D. mutation in a full length cDNA for the normal LDL receptor that could be transfected and expressed in cell cultures, and thus were able to demonstrate conclusively that such a substitution was sufficient to produce the internalization-defective phenotype. In addition, they showed (i) that the disruptive effect of the substitution was not due to the loss of the hydroxyl group of the tyrosine because the substitution of a phenylalanine at this position did not affect

internalization and (ii) that the defect was not due to disulphide bond formation between cysteine 807 and the naturally occurring cysteine at position 818, since replacement of the latter cysteine with alanine affected neither the internalization of the normal receptor nor the block in internalization produced by the cysteine 807 substitution. They concluded that this cys 807 mutation probably produces an altered three-dimensional structure of the cytoplasmic tail, thereby preventing these receptors from binding to the proteins which would normally link them to coated pits.

Very recently, the structural features in the cytoplasmic domain of LDL receptors which are required for the targeting of the receptors to coated pits, were determined (52). Twenty-four mutations in this domain were produced by oligonucleotide-directed mutagenesis. Biochemical analyses of these showed that the first 22 amino acids of the cytoplasmic domain (residues 790-811) are sufficient for rapid internalization, with the amino acid at position 807 being especially critical. Aromatic residues (tyrosine, phenylalanine or tryptophan) at this position allow rapid internalization, with charged or uncharged aliphatic residues being unable to substitute. Such specificities indicate that the region of the cytoplasmic domain which is adjacent to the plasma membrane may participate in protein-protein interactions that facilitate LDL receptor clustering in coated pits. Another striking finding was that none of the mutations (either produced in this study, or as observed previously in FH patients) interfered with the recycling of

the LDL receptors; it would seem that all forms of the LDL receptor that enter coated pits, are also recycled.

### 1.3.3 Mutations involving recombinations between Alu sequences.

The human genome contains repetitive DNA sequences, known as "Alu sequences". On average, these occur once in every 5 000 bp in the human genome; they are present frequently in intervening sequences, and are removed by splicing during mRNA processing (218). Alu sequences are approximately 300 bp in length, and consist of two head-to-tail repeats, referred to as the left and right monomeric units (57); the right monomeric unit is longer than the left unit due to a 30 bp insertion. Sequence analysis has revealed that the Alu repeats have many of the characteristics of transposable elements and pseudogenes (218). In addition, they are often flanked by direct repeats, and have A-rich sequences at their 3' ends.

#### (a) Intra-strand recombinations.

Lehrman et al (142) have reported the molecular basis of a deletion that produces a null phenotype at the LDL receptor locus, using the mutant null allele from a patient known as FH 381 (the mother of J.D.). This mutation is produced as the result of a 5 kb deletion: the Alu sequence in intron 15 has been joined to an upstream sequence in exon 13 (instead of recombining with a downstream sequence). The

unexpected stretch of complementarity between the Alu sequence and the coding sequence in exon 13 facilitates the formation of a double stem-loop structure. Similar double stem-loop structures can be drawn for other known deletions, eg. the deletion in the beta-globin gene cluster. This raises the possibility that such structures may predispose to large deletions in the human genome.

A mutant allele at the LDL receptor locus, bearing a deletion of one of the seven repeats, (FH 626), has been described by Hobbs et al (104). Molecular cloning was used to show that homologous recombination between repetitive Alu sequences in intron 4 and intron 5 of the gene produced a deletion which removed exon 5 (which normally encodes for the sixth repeat of the ligand binding domain). The resultant mRNA - in which exon 4 is spliced to exon 6, thus preserving the reading frame - produced a shortened protein that reached the cell surface and reacted with anti-receptor antibodies, but did not bind LDL (this ligand contains only apo B-100: section 1.2.3). Beta-VLDL, a lipoprotein type that contains both apo E and apo B-100, was still able to bind to the deleted protein. Such data indicate that the sixth repeat is required for LDL binding but not for that of beta-VLDL. In addition, these data support the hypothesis that the seven repeated sequences in the receptor constitute the LDL receptor binding domain.

Genomic cloning and DNA sequencing techniques have recently been used to identify the nature of the LDL receptor gene mutation in a Japanese FH homozygote patient (FH 781) (143).

Recombination between the Alu elements in intron 15 and exon 18 of the LDL receptor gene have produced a deletion which joins intron 15 to the middle of exon 18 (this exon encodes the 3' untranslated region) (Fig. 1.4A), thus removing all the 3' splice acceptor sites distal to intron 15. A truncated receptor is thus formed, which lacks the normal membrane-spanning region and cytoplasmic domain, and which has 55 abnormal amino acids at its COOH terminus. More than 90% of these receptors are secreted from the cells; those remaining on the surface show defective internalization, as has been previously reported (165, 232). The mechanism(s) by which some of these receptors remain attached to the cell surface is, as yet, unknown.

To date, four different mutant LDL receptor genes, which originate from intra-strand recombination between Alu sequences, have been identified (as reviewed above, and as summarized in Fig. 1.4A). A total of seven Alu sequences are involved in these deletions (illustrated in Fig. 1.4B). The breakpoints are apparently not randomly located - six of the seven occur in the left arm, in the region between the A and B sequences that are homologous to the internal promoters found in genes transcribed by RNA polymerase III (181). It is worth noting that Alu sequences are also apparently involved in four deletions of the globin gene complex (Fig. 1.4B); three of the four breakpoints have been shown to occur in the left arm, and two of these are found between the A and B sequences (100, 114, 177, 250). Lehrman et al (143) have speculated that the tendency for

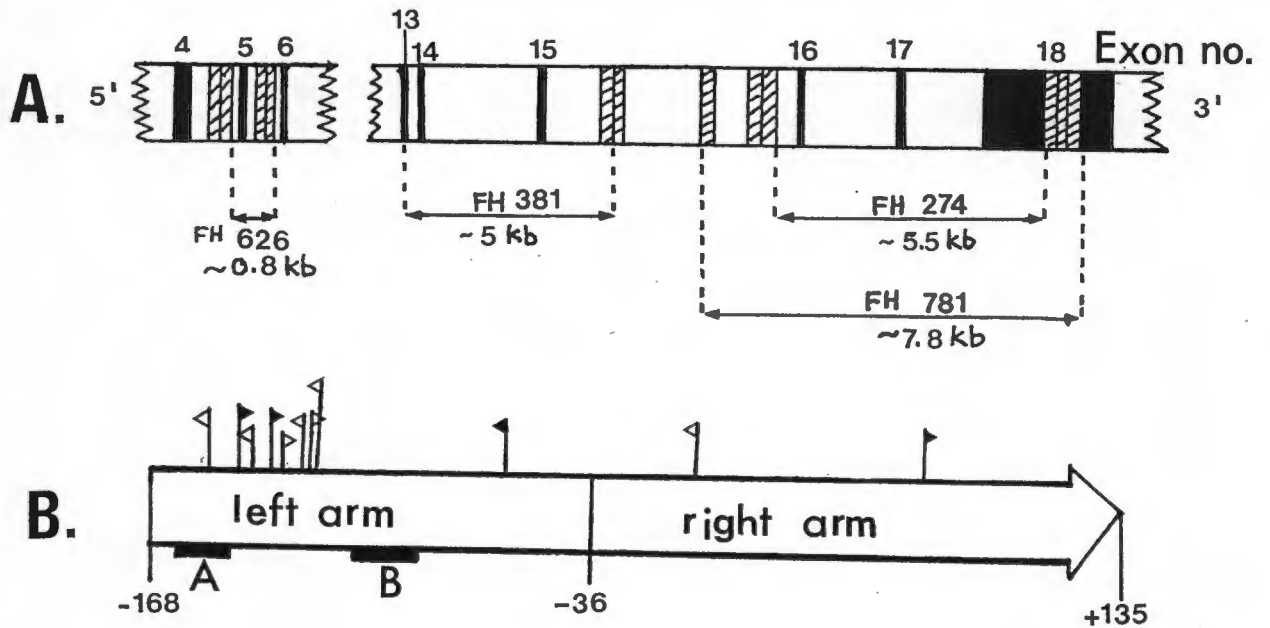


Fig. 1.4: (A) Intra-strand deletions involving Alu sequences in the LDL receptor gene.

Portions of the LDL receptor gene are shown schematically, with exons, introns and Alu repeats represented by closed, open and striped segments, respectively. The horizontal arrows indicate the four deletions described in sections 1.3.2 and 1.3.3(a).

(B): Clustering of (intra-strand) deletion endpoints within the consensus Alu repeat for mutations in the human LDL receptor and the globin genes.

A consensus Alu repeat (57) is drawn with left and right arms indicated. The locations of nine deletion joints are noted, and the direction of each deletion is indicated by the closed (globin deletions) or open (LDL receptor deletions) arrowheads. Regions of the Alu repeat that correspond to the A and B sequences of RNA polymerase III promoters (181) are shown by the solid bars.

Adapted from reference 143.

most of the deletion breakpoints to occur between the A and B sequences of the left arm (in the cases of the LDL receptor gene and the globin gene complex), may indicate that these sequences are unwound/bent during transcription by RNA polymerase III (and associated transcription factors), and, in so doing, the potential for recombination is created. These data suggest that genetic deletions may frequently be caused by Alu sequence recombinations [although it is known that this is not always the case, (eg. 175)]. Further molecular genetic analysis of the large number of mutations at the LDL receptor locus should clarify this.

**(b) Inter-strand recombinations.**

Evidence to support a model in which homologous recombination between Alu repetitive sequences in introns leads to exon duplication during the evolution of proteins, is provided by the molecular analysis of a subject (FH 295) with an unusual LDL receptor gene defect. Lehrman *et al* (141) have established that the abnormally long LDL receptor precursor (170 kd instead of 120 kd) produced by this defective gene, resulted from a 14 kb duplication encompassing exons 2 through to 8: unequal crossing-over occurred between homologous Alu sequences in intron 1 and exon 8, to produce a mutant receptor bearing 18 continuous cysteine-rich repeat sequences instead of the normal nine. (Seven of the duplicated repeats are derived from the ligand-binding domain, and two repeats are part of the EGF factor precursor homology region). The apparent mwt of the

170 kd precursor increased to 210 kd after normal carbohydrate processing. On the cell surface, the receptor bound reduced amounts of LDL and underwent inefficient internalization and recycling.

Horsthemke et al (107) have recently described a mutant LDL receptor gene which bears a 4 kb deletion (using cells obtained from patient T.D.) It is thought that the deletion arises from unequal crossing-over between two Alu sequences that are in the same orientation: one in intron 12, the other in intron 14. The fusion of exons 12 and 15 alters the reading frame of the mRNA, thereby creating a premature stop codon. This mRNA would specify a truncated receptor protein lacking the C-terminal 100 amino acids of the EGF precursor-like region, as well as the O-linked sugar, transmembrane and cytoplasmic domains. Immuno- and ligand-blot analyses have failed to detect any truncated protein.

#### 1.4 Experimentally induced mutations in the LDL receptor.

The power of biochemical-genetics in studying a complex multicompartmental cellular pathway - such as receptor-mediated endocytosis - is obvious, but there do exist severe restrictions to the genetic analysis using cells derived from human patients. For example, the variety of naturally occurring human mutations is limited, and these mutations must be compatible with the growth and survival of the entire organism. To circumvent these problems, techniques have been developed to isolate cultured mammalian somatic

cell mutants with defects in receptor-mediated endocytosis. Krieger and co-investigators have devised two methods for isolating mutants with defects in the endocytosis of LDL, and a nutritional selection procedure to detect the revertants of such mutants (reviewed in reference 134). Using these techniques, they have isolated a large collection of LDL receptor-deficient CHO cell mutants, and several revertants of these mutants. The mutants define at least four genetic complementation groups [known as Idl A, Idl B, Idl C and Idl D - see (134)], which probably represent four distinct genes required for normal LDL receptor function.

Studies performed with polyclonal antibodies on the collection of Idl A mutants, showed the existence of multiple forms of the receptor in these mutants, confirming that the Idl A locus is the structural gene for the LDL receptor (133) (thus the Idl A mutants are analagous to cells derived from FH patients). Further antibody studies also revealed that the LDL receptor deficiencies in Idl B, Idl C and Idl D mutants (which all produce mature LDL receptors that are abnormally small and heterogeneous in size) are associated with general defects in processing N-linked, O-linked and lipid-linked carbohydrate chains (126). Kingsley et al (125) recently used Idl D cells to show that O-linked chains may be crucial for receptor stability.

It is anticipated that the study of such mutants will provide further valuable insight into the structure and functioning of the LDL receptor - knowledge which has thus

far been derived mainly from studies of naturally occurring human mutations.

### 1.5 Regulation of cellular cholesterol homeostasis.

All animal cells require cholesterol as a structural component of plasma membranes. Cellular cholesterol content is regulated through the integration of two pathways that govern the supply of exogenous and endogenous cholesterol, both of which are controlled by end-product repression (for review, see 86). Under conditions of cholesterol deprivation, cells synthesise large amounts of mRNA for the LDL receptor; the increased expression of this receptor facilitates the uptake of exogenous cholesterol by receptor-mediated endocytosis (32, 199). In addition, cells also increase their endogenous cholesterol production by increasing the amount of mRNA for two sequential enzymes in de novo cholesterol biosynthesis, namely 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase (79) and HMG CoA reductase (86).

All three of these mRNAs are strongly repressed when cholesterol accumulates intracellularly, thereby limiting the uptake and synthesis of this compound (40, 78, 192, 199). Relatively little is known about the mechanisms of feedback suppression of mRNA production in higher eukaryotes compared to what is known for prokaryotes. In 1985, Osborne et al (176) established that the sequences responsible for the promotion and inhibition of transcription of the HMG CoA

reductase gene are located within 300 bp of the 5' side of the transcription initiation sites. Sudhof et al (229), working on the LDL receptor gene, have recently published evidence in support of a model in which a single 42 bp element contains a sequence that binds a positive transcription factor. It is conceivable that transcription is repressed by sterols due to inactivation of this positive transcription factor. Alternatively, it is possible that sterols may bind to a protein which, in turn, binds to this 42 bp element, thereby preventing the positive transcription factor from binding. Kandutsch (118, 119) has postulated that repression of HMG CoA reductase could be mediated by the binding of intracellular oxysterol metabolites (such as 25-hydroxycholesterol or 7-ketocholesterol) to a cytosolic protein; this sterol-protein complex could then inhibit the transcription or translation of the HMG CoA reductase gene. In support of this model, a cytosolic binding protein, specific for oxysterols, has been identified (119, 239).

#### 1.6 Receptor and ligand degradation.

The degradative process, or any step which commits a receptor to this pathway, would be one of the logical sites at which to regulate receptor level (the absolute level of receptor is determined both by its rate of synthesis and its rate of degradation). Receptors and ligands may, or may not, share the same fate after entry into the cell, and in some cases - particularly those where recycling occurs - the receptors escape the final destiny of the ligands whose

uptake they mediate (namely, lysosomal degradation). For example, recent studies with the F<sub>2</sub> receptor and mannose 6-phosphate receptor suggest that these proteins are degraded intralysosomally, together with their ligands (162, 253), as is the case for the EGF receptor (20, 67). On the other hand, various investigators (131, 135), using the heavy isotope density-shift method, have shown that insulin and the insulin receptor traverse different pathways for degradation.

It has been suggested that ligand dissociation from internalized receptors, within an acidic vesicle, plays a critical role in allowing the receptor to undergo recycling, perhaps by providing a signal that targets receptors to be recycled (30). Recently, Huecksteadt et al (108) have published results which are contrary to this proposal: these investigators covalently attached iodinated, photoreactive insulin to insulin receptors on the cell surface of isolated rat adipocytes, and were able to show that such covalently occupied receptors recycled at rates comparable to rates observed for native insulin/insulin-receptor complexes (108). It would therefore appear that the physiological dissociation of insulin from internalized receptors may represent an independent event, not casually related to the steps governing insulin receptor recycling; such processes may also be true of other receptor recycling systems.

As an alternate approach to address the problem of receptor degradation, Schwartz et al (215) examined the fate of the

asialoglycoprotein (ASGP) receptors on Hep G2 cells following exposure to polyclonal antibodies directed against the receptor. They showed that the antibody induced a rapid cellular loss of the ASGP receptors; addition of the weak base, primaquine, blocked this loss, while the thiol protease inhibitors, leupeptin or EC 64, inhibited the lysosomal degradation of the ligand but not the receptors. Thus, the pathway and/or mechanism(s) responsible for the antibody-induced ASGP receptor loss are apparently distinct from the lysosomal ligand breakdown.

The somewhat complex field of general receptor and ligand breakdown has been comprehensively reviewed by Van der Westhuyzen and Coetzee (247). Other aspects of receptor degradation are reviewed in Sections Four and Five of this thesis.

### 1.7 Conclusion.

The study of the LDL receptor function and metabolism has been an exciting and very valuable one in several ways. It has served as a prototype for the elucidation of the process of receptor-mediated endocytosis. Analyses of these receptors have provided much insight regarding the mechanisms by which genes regulate cholesterol metabolism. This receptor is of significant clinical importance since mutations occurring in the gene for the receptor disrupt the regulation of blood cholesterol levels, producing hypercholesterolemia and atherosclerosis. The processes

involved in the uptake and degradation of the ligand, LDL, have been characterized, and the accompanying regulation of cellular cholesterol homeostasis is well defined. Extensive studies have yielded much information about the synthesis and structure of the receptor itself (reviewed in 34 & 88). The mechanism(s) by which these important receptors are segregated into degradation pathways (a routing apparently distinct from ligand delivery to lysosomes) have not been delineated to date. The study reported in this thesis is focused mainly on the cellular degradation of the low density lipoprotein receptor, and is contrasted with that of its ligand, LDL.

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## 2.1 Introduction.

Extensive studies, (performed mainly by M.S. Brown, J.L. Goldstein and coworkers - see Section One for literature review) on cultured HSF, as well as other cell types, have led to the elucidation of the pathway by which these cells take up and metabolize LDL (33, 34). The concept of "receptor-mediated endocytosis" was formulated in 1974 (84) to explain the observation that regulation of cellular cholesterol metabolism depended on the sequential cell surface binding, internalization and degradation of plasma LDL. This postulate has been verified by further biochemical, eg. (2), and morphological, eg. (4), studies.

There are four general properties that collectively define receptor-mediated endocytosis (82):

(i) The cell surface binding component is a molecule which binds a specific exogenous ligand, thereby generating a physiological effect. (Note: there are other cell surface components which function as binding sites for opportunistic foreign molecules, eg. viruses; these are not classed as receptors).

(ii) Ligand binding and internalization are, effectively, coupled events; it has been shown that the half-time of internalization for a range of receptor-ligand complexes is less than 10 minutes (8, 83, 242).

(iii) Receptor-bound proteins enter cells through coated pits; receptors may localize spontaneously in these regions, or ligand binding may induce receptor migration to these areas (2, 157).

(iv) Internalized proteins are usually delivered to lysosomes, the site of their subsequent degradation to amino acids (2, 242).

The well-characterized LDL receptor system in cultured HSF exhibits each of the above characteristics of receptor-mediated endocytosis.

In 1976, Kayden et al (121) used long-term lymphoid cells, obtained from human lymphocytes, to study the regulation of cholesterol biosynthesis, and to show that genetic abnormalities in this regulation are expressed in these cells. They reported that the cells responded to LDL in a manner similar to HSF: human LDL, but not HDL, suppressed HMG CoA reductase activity and stimulated cholesterol esterification. These effects were obtained using LDL at concentrations similar to those used in the fibroblast lines. Furthermore, lymphoid cells derived from individuals with homozygous FH showed neither suppression of HMG CoA reductase activity, nor stimulation of cholesteryl ester formation when incubated with LDL (121).

Ho et al (103) subsequently used similar established human lymphoid cells from a normal subject to confirm that these cells did indeed possess LDL receptors. Binding of LDL to these receptors was followed by internalization of the receptor-ligand complexes, with subsequent degradation of the lipoprotein. Lymphoid cells from a patient with the homozygous form of FH did not bind, internalize or degrade <sup>125</sup>I-LDL via any high-affinity (receptor-mediated) processes.

Alternate methods to introduce experimentally labeled LDL into cells (ie. apart from receptor- and non-receptor mediated processes on intact cells), can be drawn from techniques currently used to incorporate macromolecules into the cytoplasm of cultured cells (61, 161) - an essential tool in developing biological fields such as the analysis of cytoplasmic protein degradation. Ideally, methods used for loading macromolecules should be simple and applicable to a variety of cell types; they should be able to load a wide range of macromolecules into a significant proportion of the target cell population, to a measurable extent. Macromolecule loading should, initially, be cytoplasmic, and cell function, morphology and viability should not be extensively compromised by the procedure.

Several methods are available for loading, each of which is known to possess certain disadvantages. Microneedles may be used to inject substances into individual cells (58); the procedure, however, is a tedious one, in which it is difficult to control the amount of macromolecule injected, and usually only a limited number of cells are actually loaded. Alternately, liposomes (178) or red blood cells (80, 204), containing trapped substances, can be fused with host cells, thereby delivering the substances into the host cytoplasm. Artifacts may be encountered in studies of the membrane functioning of cells loaded in this way, since the membrane of the loaded cell is contaminated with red blood cell or liposome membrane. Loading by hypo-osmotic shock (170) bears the disadvantage that pinosomes containing the

macromolecules to be loaded are also subject to osmotic lysis, and thus may release their macromolecules into the cytoplasm. In addition, not all of these pinosomes containing molecules to be loaded, are lysed by the osmotic shock.

McNeil et al (161) recently described a method for loading macromolecules into the cytoplasm of adherant cells, which involves scraping the cells, thereby mechanically perturbing the plasma membrane. They propose that transient holes (probably about 50 nm in diameter) are opened in the plasma membrane at the sites of tightest cell adherence to the plastic substratum; exogenous macromolecules would conceivably enter the cytoplasm by diffusion through these holes (161). These investigators have shown that, by varying the concentration of the macromolecules present during scraping, and by sorting scrape-loaded cells in a flow cytometer, cell populations which are loaded to a known extent, may be selected for.

In this section, long-term lymphoid (lymphoblastoid) cell lines, derived from normal subjects, were used to examine the fate of  $^{125}\text{I}$ -LDL subsequent to its uptake via receptor-mediated endocytosis. These results were compared with data available from the well-studied HSF system. Lymphoblastoid cells are particularly suitable for detailed in vitro studies of lipid metabolism since they proliferate rapidly, are apparently permanent and they offer potential advantages in studies where large volumes of cellular material are needed.

Finally, the degradation of ligand taken up in intact cells via receptor-mediated endocytosis on LDL receptor normal HSF was contrasted with the degradation of ligand introduced via scrape-loading into the cytoplasm of LDL receptor negative fibroblasts.

## 2.2 Experimental procedures.

### 2.2.1 Cells.

Routine tests were performed to check that all tissue culture media were pyrogen-free. The cell cultures were all free of mycoplasma, as determined by the BRL Mycotect Mycoplasma Detection system (Bethesda Research Laboratories, Maryland, USA).

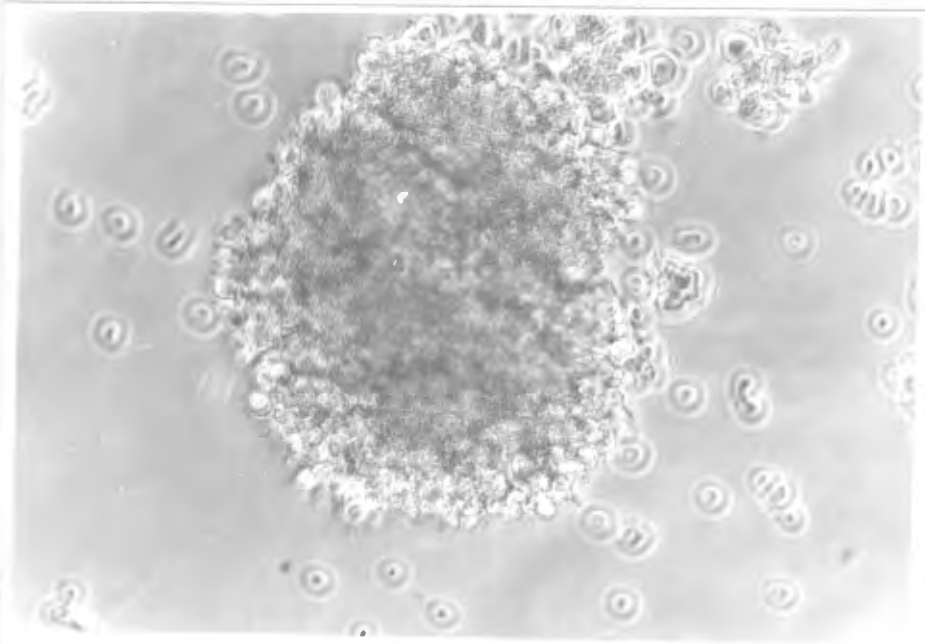
#### Lymphoblastoid cells.

Lymphoblastoid cells were prepared from human lymphocytes (248). In brief, medium containing Epstein-Barr virus (EBV) was prepared from semi-confluent cultures of the virus-producing marmoset cell line, B 95/8 (163). Fresh Ham's F10 medium containing 15% bovine fetal calf serum (FCS) was added to the cells for 4 days, after which it was removed, filtered through a 0.45  $\mu\text{m}$  membrane, and mixed with an equal volume of fresh Ham's F10/FCS medium. This mixture constituted the transformation medium, and was effective for at least 3 months when stored at 4°C.

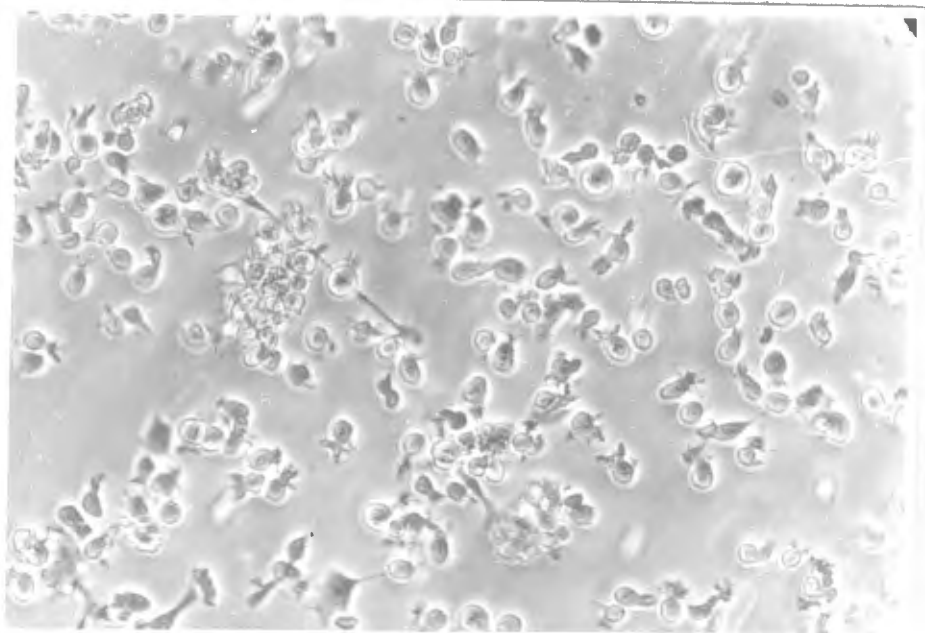
Lymphocytes were prepared from 10 to 20 ml of blood, using either heparin or EDTA as the anticlotting agent. The blood was kept on ice for up to 24 hours before use. Aliquots of blood (10 ml) were layered above Histopaque solutions (5 ml) and centrifuged (1 500xg, 20 mins). The white cell band was removed and washed once with Ham's F10 medium (10 ml), resuspended in transformation medium (3 ml), transferred to a 25 cm<sup>2</sup> tissue culture flask, and incubated in a humidified incubator (95% relative humidity, 5% CO<sub>2</sub>). Small volumes of medium were added, generally on alternate days, until rapid multiplication of transformed lymphoblasts allowed passage into 75 cm<sup>2</sup> flasks. This process generally took between 1 and 3 weeks.

The transformed cells were routinely maintained in suspension culture at densities between  $0.2 \times 10^6$  and  $1.0 \times 10^6$  cells/ml, by discarding about 75% of the medium (and hence a corresponding proportion of the cells), and adding a similar volume of fresh medium to the remaining cells in the culture flask. The medium used for long-term propagation of the cells consisted of Eagle's minimal essential medium buffered with Earle's salts (MEM), supplemented with 10% heat-inactivated FCS (HIFCS), 10% tryptose phosphate broth, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. Actively growing healthy cultures existed as mixed suspensions of large clumps of cells, as well as dispersed cells (Fig. 2.1A). The clumps were easily broken up by aspirating gently through a pipette several times (Fig. 2.1B).

A.



B.



**Fig. 2.1: Phase contrast micrographs of a cultured LDL receptor normal lymphoblastoid strain.**

**A:** A healthy, actively growing suspension culture, comprising clumps of cells, as well as dispersed cells.

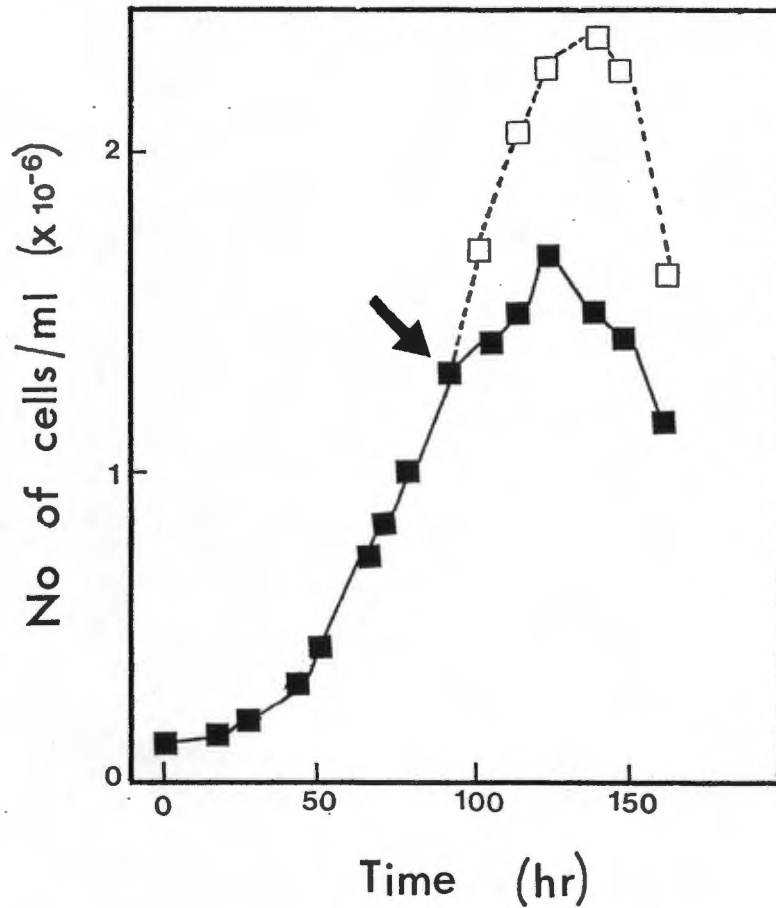
**B:** After gentle aspiration through a pipette several times, the cell clumps become dispersed.

**Magnification: X 200.**

To store the cells for subsequent use, cultures in the logarithmic growth phase were resuspended in medium containing 10% glycerol at a concentration of approximately  $0.5 \times 10^6$  to  $1 \times 10^6$  cells/ml. 1 ml aliquots were frozen under liquid nitrogen vapour ( $-60^\circ\text{C}$ , 30 mins) before final storage under liquid nitrogen. For re-use, the cells were thawed rapidly in a  $37^\circ\text{C}$  water bath. The cells were then pelleted by centrifugation (2 000 rpm, 3 mins), washed once in 10 ml of medium to ensure complete removal of the glycerol, and resuspended finally in 4 ml medium. Hereafter, cell growth and maintenance were carried out as detailed for the newly transformed cultures.

A comparison of cell growth in cultures which had been routinely disturbed, ie. cell clumps dispersed to single cell suspensions by aspiration (5 to 10 passages through an automatic pipette-man) twice daily, with cultures where growth was undisturbed until the time of measurement, showed no significant differences in growth rate (data not given). Subsequently, lymphoblastoid cell cultures were routinely grown and maintained in  $75 \text{ cm}^2$  tissue culture flasks without disrupting the cell clumps unnecessarily.

The doubling time of these cells in medium containing 10% HIFCS was about 25-30 hours. Cell density was found to plateau between  $1.5 \times 10^6$  and  $2.0 \times 10^6$  cells/ml (Fig. 2.2). If, during growth, the cells were spun down and resuspended in an identical volume of fresh medium, cell growth was markedly increased compared to cultures where the medium had not been changed (Fig. 2.2). Cell numbers were always



**Fig. 2.2: Growth characteristics of cultured normal human lymphoblastoid cells.**

Data are the mean values of duplicate flasks seeded at  $0.1 \times 10^6$  cells/ml, and maintained in culture medium as described in section 2.2.1. The arrow marks the point at which the cells from one set of flasks were pelleted and resuspended in an identical volume of fresh medium (□), while the cells in the remaining set of flasks were given no medium change (■).

measured by counting aliquots of dispersed cultures in a coulter counter, Model Zf. Identical growth characteristics were obtained using total cell protein as a means to assess cell proliferation (data not shown). The cultures were inspected daily under the phase-contrast microscope to check for the visible signs of good growth - namely, healthy, translucent appearing cell clumps. In healthy cultures, the cells on the edges of the clumps, as well as the single cells, showed distinct "pseudopodia-like" projections. These projections were observed to move gradually when cultures were watched continuously, for a period of several minutes, under the light microscope.

#### Human skin fibroblasts.

The fibroblast strains used were GM 3348A, (characterized as LDL receptor normal), and LVS, (characterized as LDL receptor negative); GM 3348A was obtained from the Human Genetic Mutant Cell Repository (Camden, N.J., USA), and LVS was established in our laboratory from an FH homozygote (reference 248, kindred 3b in that study). Monolayer cultures were grown in 75 cm<sup>2</sup> flasks and maintained in a humidified incubator (95% relative humidity, 5% CO<sub>2</sub>) at 37°C. Each flask contained approximately 12 ml of growth medium consisting of Dulbecco's Modification of Eagle's Minimum Essential Medium, (DMEM), supplemented with 10% HIFCS, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. Confluent monolayers, between the twelfth and nineteenth passages, were dissociated with 5 ml 0.05% trypsin/0.02% EDTA solution for 7 mins at 37°C, and were subsequently seeded (day 0) into 60 mm Petri dishes at a

concentration of  $0.1 \times 10^6$  cells per dish (in 4 ml growth medium/dish). Unless otherwise stated, cultures in the late logarithmic growth phase (day 5), were used. Cells were inspected daily under phase-contrast microscope optics; cells from semi-confluent monolayers always appeared spindle-shaped (Fig. 2.3).

Cells were stored for later use as follows: the trypsin/EDTA-dissociated cultures were resuspended at about  $1 \times 10^6$  cells/ml in medium containing 10% dimethylsulphoxide. 1 ml aliquots were frozen in nitrogen vapour, prior to being stored under liquid nitrogen. For re-use, the frozen cells were thawed rapidly in a  $37^\circ\text{C}$  water bath, and the cell suspensions were transferred to  $75 \text{ cm}^2$  stock flasks containing 12 ml growth medium. This medium was replaced with the same volume of fresh medium the next day, in order to remove the dimethylsulphoxide.

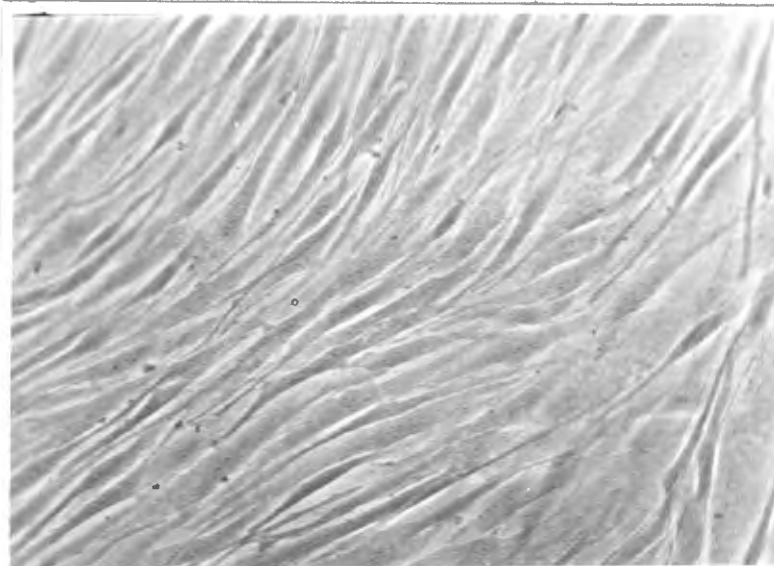
#### 2.2.2 Preparation of lipoproteins and lipoprotein-deficient serum.

Human LDL (density  $1.019-1.063 \text{ g/ml}$ ) and human lipoprotein-deficient serum (LPDS) (density  $> 1.25 \text{ g/ml}$ ) were isolated from the plasma of normolipidemic donors by ultracentrifugation, using a Beckman L8-70 ultracentrifuge. Fractions were isolated by discontinuous gradient centrifugation using the method of Chung *et al.* (41). A discontinuous NaCl/KBr density gradient was formed as follows. The density of the plasma [containing 0.01% EDTA

A.



B.



**Fig. 2.3: Phase contrast micrographs of a cultured LDL receptor normal human skin fibroblast cell line (GM 3348A). The cells were photographed at various times after seeding as described in section 2.2.1.**

**A: Sub-confluent layer (day 3 after seeding), showing spindle-shaped cells.**

**B: Confluent growth in monolayer.**

**Magnification: X 200**

and the protease inhibitors aprotinin (0.4%, v/v) and phenylmethylsulphonyl fluoride (PMSF) (0.2 mM)] was adjusted to 1.3 g/ml with solid KBr, and normal saline (0.9% NaCl - 0.01% EDTA, pH 7.0, density = 1.006 g/ml) was layered over the adjusted plasma. Gradients were centrifuged (50 000 rpm, 2.5 hours, 10°C) in a VTi 50 rotor; in each polyallomer tube, the various lipoproteins resolved in well-defined bands, corresponding to their specific density ranges, with VLDL and intermediate density lipoproteins (IDL) at the top, followed by LDL, then HDL, and finally LPDS in the lower portion. The LDL fractions were recovered, pooled and washed at a density of 1.063 g/ml by an overnight centrifugation at 50 000 rpm in a Ti 60 rotor. The yellow LDL-containing layer at the top of each of the tubes was collected and these were pooled.

The bottom fraction obtained after the initial 2.5 hour centrifugation was adjusted to 1.25 g/ml with solid KBr, and was spun in a VTi 50 rotor (50 000 rpm, 6-8 hours). The residual HDL, which was contained in a yellow layer at the top of each tube, was removed, and the LPDS fraction was retained.

Both the LDL and LPDS fractions were dialysed extensively against 0.9% NaCl-0.01% EDTA, pH 7.4, at 4°C. After dialysis, 2 units/ml of thrombin was added to the LPDS; this fraction was then kept at 4°C for 24 hours, after which it was centrifuged in a JA 20 rotor (20 000 rpm, 10 mins, 10°C), and the supernatant kept.

An alternate, shorter method of LPDS preparation, which did not involve the concurrent isolation of LDL, was sometimes used. Briefly, the procedure was as follows. The density of the plasma (containing 0.25 M EDTA) was increased to 1.215 g/ml, and the adjusted plasma was subsequently spun in a Ti 60 rotor (59 000 rpm, 36 hours, 4°C). VLDL, LDL and HDL fractions banded in the top portion of each ultracentrifuge tube, and were discarded. The lipoprotein-deficient fraction, which remained as a separate yellow layer in the lower portion of each tube, was pooled, dialysed against normal saline, and processed further according to the method detailed above.

The LDL and LPDS fractions were sterilized by filtration through a millipore filter (0.45  $\mu$ M pore diameter). Heat inactivation of the LPDS was performed to remove complement by incubating this fraction for 30 mins in a 56°C water bath. The protein content of both the LPDS and LDL fractions was determined by the method of Lowry et al (147) using bovine serum albumin (BSA) as standard. Protein concentrations of the preparations were generally in the following ranges: LDL, 8-20 mg protein/ml and LPDS, 50-80 mg protein/ml. The LPDS was frozen at -12°C until required; LDL was stored at 4°C and used within a month.

The purity of each LDL preparation was assessed by (i) lipoprotein electrophoresis using the Pharmacia FBE 3000 Electrophoretic System, and the Beckman Paragon Lipoprotein Reagent Kit and (ii) SDS-PAGE according to the method of Laemmli et al (137) on linear 5-12% polyacrylamide gradients

containing glycerol, and a 3% stacking gel. Electrophoresis according to (i) showed that the LDL preparations were essentially free of other human lipoproteins. The second system, (ii), demonstrated the presence of an apolipoprotein B-100 band (apparent mwt about 340 000 daltons), contaminated only very slightly with minor bands (< 1%), and was thus essentially free of other plasma proteins.

### 2.2.3 <sup>125</sup>I-labeling procedure for LDL.

Iodination of LDL was performed using the iodine monochloride procedure of MacFarlane, as modified by Bilheimer et al (27). In most cases, 5 mCi sodium iodide (<sup>125</sup>I) was used to iodinate 5 mg LDL, after which the radiolabeled LDL was dialysed for 24 hours at 4°C against several changes of 0.9% NaCl containing 0.01% EDTA, pH 7.4, to remove free iodine. The label in LDL was 99% precipitable with 5% (w/v) trichloroacetic acid (TCA), and lipid radioactivity, as assessed after extraction by the method of Folch et al (71), was approximately 3%. Specific activities of the <sup>125</sup>I-LDL preparations ranged from about 300-700 cpm/ng LDL protein. The labeled LDL was always used within 2 weeks of iodination. Immediately prior to use, the <sup>125</sup>I-LDL was spun briefly in a microfuge (11 000xg, 1 min) to remove any particulate matter.

#### 2.2.4 Acetylation of LDL

Acetylated LDL (AcLDL) was produced according to the method of Fraenkel-Conrat (72). An ice-cold saturated sodium acetate solution was added to LDL in a 1:1 (v/v) ratio. This was gently mixed, and then kept on ice for 5 minutes before adding acetic acid (1  $\mu$ l acetic acid /mg LDL). The acetic acid was added over a period of an hour, with mixing after every addition. The solution was subsequently extensively dialysed as described in section 2.2.3, followed by a brief centrifugation (11 000xg, 1 min) to remove any precipitated protein. As measured by the Lowry method (147), the acetylation procedure resulted in a 10-20% loss of lipoprotein protein.

Acetylated LDL was iodinated as described in section 2.2.3; the specific activity of these labeled lipoproteins was about 250-300 cpm/ng protein.

#### 2.2.5 Upregulation of LDL receptor activity.

Unless otherwise stated, LDL receptor activity was upregulated in cell cultures prior to performing each experiment. The method used was essentially that described by Brown and Goldstein (32), and entailed the incubation of cells in medium containing LPDS instead of FCS. The cells were always > 95% viable, as assessed by trypan blue dye exclusion, at the initiation of experiments.

### **Lymphoblastoid cells**

The cells were pelleted by centrifugation (2 000xg, 3 mins, room temp), resuspended in phosphate buffered saline (PBS) (pH 7.4), and then spun again. Final resuspension of the pellets was in MEM containing 5 mg LPDS protein/ml (MEM/LPDS), 60  $\mu$ g/ml penicillin G and 100  $\mu$ g/ml streptomycin sulphate, at  $1 \times 10^6$  cells/ml. The LPDS containing medium was changed after 24 hours. Unless otherwise specified, upregulation was performed for 48 hours.

### **Human skin fibroblasts**

Monolayers were washed once with PBS (usually on day 5 of cell growth), and incubated with DMEM containing 5 mg protein/ml LPDS (DMEM/LPDS), 60  $\mu$ g/ml penicillin G and 100  $\mu$ g/ml streptomycin sulphate for 48 hours, unless otherwise indicated. After 24 hours, the upregulation medium was replaced with 3 ml/dish of fresh upregulation medium.

### **2.2.6 $^{125}$ I-LDL metabolism, after incubation with cells at 37°C.**

#### **Lymphoblastoid cells.**

After incubation in upregulation medium, the cells were resuspended in fresh MEM/LPDS at  $2 \times 10^6$  cells/ml, and dispensed in 35 mm culture dishes (1 ml/dish).  $^{125}$ I-LDL, in the absence or presence of excess unlabeled LDL, was added at the required concentration to each dish; incubations were then carried out at 37°C for 4 hours. The cell

suspensions were transferred to 15 ml plastic centrifuge tubes, chilled thoroughly on ice for 5 mins, and the cells were pelleted by centrifugation (2 000xg, 3 mins, 4°C). The medium was used to determine the noniodide TCA soluble degradation products, as described below. Each cell pellet was carefully resuspended in 2.5 ml PBS containing 0.2% albumin (PBS-ALB), by aspirating twice with a disposable plastic pasteur pipette. The cells were centrifuged again, and the washing procedure was repeated twice using PBS. All washing procedures were performed at 4°C.

**Determination of surface-bound  $^{125}\text{I}$ -LDL** - the final pellet was resuspended in 1 ml of ice-cold sodium heparin solution (4 mg/ml in PBS), and incubated on ice for 1 hour, after which centrifugation (2 000xg, 6 mins, 4°C) was carried out to separate the cells from the heparin solution. This heparin-releasable radioactivity was taken as a measure of cell surface bound  $^{125}\text{I}$ -LDL.

**Determination of intracellular  $^{125}\text{I}$ -LDL** - 1 ml 50% aqueous methanol was added to each pellet; the cell suspension was vortexed and transferred to a glass test tube. The plastic centrifuge tube (in which the pellet had been) was then washed twice with 1 ml 100% methanol, and these washes were combined. 2.2 ml of chloroform was added to the methanol; the test tubes containing these mixtures were vortexed well, and centrifuged (2 000 rpm, 30 mins, 4°C) to produce a protein pellet. After the centrifugation, the chloroform/methanol mixture was poured off carefully, and the pellets were maintained in a 60°C water bath until dry. They were then dissolved in 1 ml 1 N NaOH and left overnight

at 37°C. The solubilized protein in each test tube was transferred to a plastic counting vial and counted for intracellular  $^{125}\text{I}$ -LDL content. The protein content in each NaOH-solubilized pellet was measured by adding 1 ml  $\text{H}_2\text{O}$  to each sample (ie. final volume = 2 ml of 0.5 N NaOH); 0.5 ml aliquots were used for the estimation of cell protein according to the method of Lowry et al (147) with BSA as standard.

**Determination of degraded  $^{125}\text{I}$ -LDL -  $^{125}\text{I}$ -degradation products of apolipoprotein B** were determined as described by Bierman et al (26). The incubation medium was brought to a final concentration of 12.5% TCA by the addition of 0.3 ml of 50% TCA, and the samples were then kept on ice for 30 minutes or longer to ensure complete precipitation. Centrifugation (2 000xg, 30 mins) was performed to obtain the TCA-soluble fraction. The free iodide in this fraction was subsequently converted to  $\text{I}_2$  by the addition of hydrogen peroxide, as follows: 20  $\mu\text{l}$  of 40% potassium iodide was added to each 1 ml of TCA supernatant solution, followed by the addition of 50  $\mu\text{l}$  of 30% hydrogen peroxide. To extract the iodine, two volumes of chloroform were added, the mixtures vortexed vigorously, and the aqueous and chloroform phases were separated by centrifugation (2 000 rpm, 5 mins). The radioactivity in the aqueous phase was counted. To control for any noncellular lipoprotein metabolism, and to obtain a blank value,  $^{125}\text{I}$ -LDL was incubated in cell-free dishes, and the radioactivity obtained after performing the assays as described above, was subtracted from that obtained in the presence of cells.

### Human skin fibroblasts.

After upregulation of the LDL receptors on the cells as described above, monolayers were incubated in fresh DMEM/LPDS, containing the required amount of  $^{125}\text{I}$ -LDL, in the absence or presence of excess unlabeled LDL. Incubations were performed for 4 hours at  $37^\circ\text{C}$  in a humidified incubator. The cells were then cooled to  $4^\circ\text{C}$ , and the medium was collected for the determination of  $^{125}\text{I}$ -lipoprotein products (as described above).

**Determination of surface bound  $^{125}\text{I}$ -LDL** - After removing the incubation medium, the cells were washed 4 times with PBS-ALB, and then 3 times with 3 ml of PBS to remove all unbound radioactivity. The entire wash procedure was performed at  $4^\circ\text{C}$ , using appropriately chilled solutions. Surface-bound  $^{125}\text{I}$ -LDL was released by incubation of the monolayers with ice-cold sodium heparin solution (4 mg/ml in PBS) for 1 hour at  $4^\circ\text{C}$ .

**Determination of intracellular  $^{125}\text{I}$ -LDL** - Subsequent to heparin treatment, the intact cell monolayers were dissolved overnight in 2 ml 0.5 N NaOH at  $37^\circ\text{C}$ . An aliquot was counted for  $^{125}\text{I}$ -radioactivity to determine the intracellular  $^{125}\text{I}$ -LDL content (ie. the radioactivity associated with the cells after removal of receptor-bound LDL). Cell protein was assayed (147) on aliquots of these NaOH-solubilized cells.

In all cases (for HSF and lymphoblastoid cells) the data presented for surface-bound, intracellular or degraded  $^{125}\text{I}$ -LDL are high-affinity values (unless otherwise specified).

These were calculated by subtracting the values obtained in the presence of excess unlabeled ligand (non-specific component) from that obtained in its absence (total).

### 2.2.7 Cell-associated $^{125}\text{I}$ -LDL.

The term "cell-associated  $^{125}\text{I}$ -LDL" refers to the radiolabeled ligand associated with the cells, including that which is receptor-bound. In this section, cell-associated values were always obtained after incubating the  $^{125}\text{I}$ -ligand with the cells at 4°C; in some other experiments (section 4.3.4 and Fig. 4.5), cell-associated radioactivity was measured after short 37°C incubations.

#### **Lymphoblastoid cells**

After incubation in MEM/LPDS, the cells were transferred from the upregulation flask(s) to centrifuge tubes and pelleted (2 000 rpm, 3 mins, 4°C). Each cell pellet was resuspended in PBS and centrifuged again, before final resuspension at about  $2 \times 10^6$  cells/ml in Eagle's minimum essential medium without bicarbonate supplemented with 10 mM HEPES (pH 7.4), 60  $\mu\text{g/ml}$  penicillin G, 100  $\mu\text{g/ml}$  streptomycin sulphate and 5 mg protein/ml LPDS (4°C). 1 ml/dish of the cell suspension was dispensed into each 35 mm culture dish and  $^{125}\text{I}$ -LDL, in the absence or presence of excess unlabeled LDL, was subsequently added at the indicated concentration to each. Incubations were performed for 2 hours at 4°C; after which the cell suspensions were washed at 4°C as described in section 2.2.6. Each pellet

was then dissolved in 2 ml 0.5 N NaOH; the radioactivity measured represented cell-associated ligand. Aliquots of these samples were used for protein determinations (147).

In the cases where a 30 minute 37°C incubation period was used to assess cell-associated  $^{125}\text{I}$ -LDL, the procedure followed was analagous to that described above, except that the initial PBS wash of the cells was at 37°C, before final resuspension in DMEM/LPDS at the same temperature. During this short incubation period,  $^{125}\text{I}$ -LDL was bound to the receptors, internalized, but no significant ligand degradation was detected (Fig. 2.7).

#### Human skin fibroblasts

After upregulation of the LDL receptors in DMEM/LPDS medium, the monolayers were chilled to 4°C by placing the dishes on a steel tray in a 4°C cold room for 10 minutes. Each cell layer was washed once with ice-cold PBS, before adding medium as described above under the heading "lymphoblastoid cells". After 2 hours at 4°C, the incubation media were removed, and the monolayers were washed, and subsequently solubilized with NaOH, as described in section 2.2.6.

#### 2.2.8 Scrape-loading of $^{125}\text{I}$ -ligand into fibroblasts.

LDL receptor negative human skin fibroblasts, (strain LVS), were seeded into 60 mm Petri dishes, and grown for 7 days in DMEM supplemented with HIFCS (note: no upregulation of the LDL receptors was performed). Immediately prior to

scraping, each dish was washed once with 3 ml of DMEM only. 2 ml of 37°C scrape medium (DMEM + the indicated amount of radiolabeled ligand) was added to the first dish; the cells were then scraped with a rubber policeman, and the cell/medium mixture was transferred to the next dish before repeating the above procedure. 3 dishes of cells were scraped with each 2 ml of radiolabel-containing medium; the combined scrape media were finally added to 5 ml of Eagle's minimum essential medium without bicarbonate, supplemented with 10 mM HEPES (pH 7.4) at 4°C. After thorough chilling on ice, the cells were pelleted (2 000 rpm, 3 mins, 4°C) and resuspended in 2 ml of DMEM + 10% HIFCS (4°C). This suspension was layered onto 8 ml of DMEM + 50% HIFCS, and then spun (2 000 rpm, 4°C, 3 min). The cell pellet was resuspended in 5 ml of DMEM + 10% HIFCS and centrifuged as described above. The cells were finally taken up in 12 ml of DMEM + 10% HIFCS (37°C), aliquoted into a 75 cm<sup>2</sup> tissue culture flask, and incubated at 37°C for 4 hr in a humidified incubator. Dissociation of the attached cells was then performed using trypsin/EDTA (5 mins, room temp) essentially as described (section 2.2.1). A sample of the trypsin/EDTA supernatant was counted in order to determine how much <sup>125</sup>I-LDL was released. The cell pellet was resuspended in growth medium, and aliquoted into 35 mm petri dishes (2 ml/dish); 3x35 mm petri dishes were usually derived from every 2 of the original 60 mm dishes. After 4 hours, the medium was removed from each dish; the cells were washed twice with PBS, once with growth medium, and finally incubated in 2 ml/dish of the latter medium. For the purposes of subsequent analyses, this was taken as the

zero time. At various times thereafter, ranging from 0 to 90 hours, the following assays were performed on each dish of cells containing scrape-loaded  $^{125}\text{I}$ -LDL:

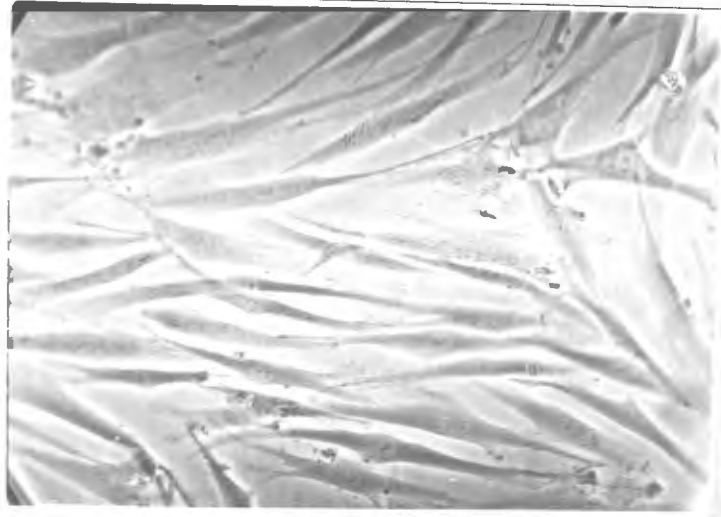
(i) The medium was removed and tested for the presence of radiolabeled degradation products, as described in section 2.2.6. In some cases (see legend to Fig. 2.11), the TCA pellet (obtained from analysis of the medium for degradation products), was measured for radioactivity.

(ii) The cells were washed twice with PBS, and then dissociated by mild trypsinization at room temperature for 5 mins. The supernatant was counted for the presence of trypsin/EDTA releasable material (surface bound).

(iii) Each cell pellet (obtained in (ii)), was washed twice with PBS at  $4^{\circ}\text{C}$  by resuspension and centrifugation (2 000 rpm, 2 mins,  $4^{\circ}\text{C}$ ). The cell pellet was dissolved in 0.5 ml of 0.5 N NaOH; the radioactivity obtained represented trypsin/EDTA resistant material. The solubilized cells were incubated overnight at  $37^{\circ}\text{C}$ , and were subsequently assayed for protein content using the method of Lowry et al (147).

The appearance of the cells was examined under phase contrast optics at various times after performing the scrape-loading (Fig. 2.4). The cells adhered to the dishes, and assumed the characteristic spindle-shaped morphology within several hours after replating. Cell viability, as assessed by trypan-blue dye exclusion, was  $> 95\%$  at each time point. In addition, total cellular protein was determined at these times (147); the results obtained were indicative of normal growth characteristics.

A.



B.

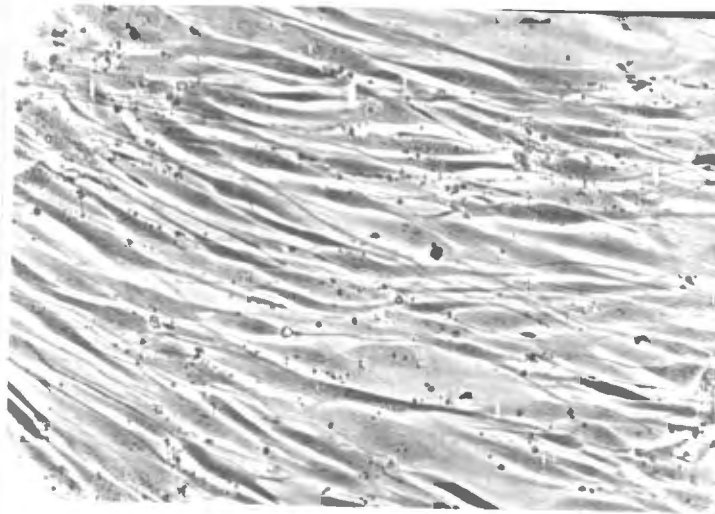


Fig. 2.4: Phase contrast micrographs of LDL receptor negative human skin fibroblast cell line (LVS), as visualized at various times after performing the scrape-loading procedure with  $^{125}\text{I}$ -LDL.

A: 24 hours.

B: 60 hours.

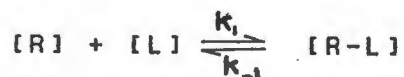
Magnification: X 200.

### 2.2.9 Calculations.

Determination of the equilibrium dissociation constant ( $K_D$ ) and binding capacity ( $B_{max}$ ).

$K_D$  and  $B_{max}$  values for the binding of LDL to the LDL receptors of lymphoblastoid cells were determined using data from studies in which  $^{125}I$ -LDL was bound to cells at the following concentration range: 1-20  $\mu\text{g/ml}$  ( $4^\circ\text{C}$ ). At each lipoprotein concentration used, the receptor-bound (high-affinity) value was calculated as described (section 2.2.6).

Equilibrium binding of LDL to the cell surface LDL receptor is only achieved at  $4^\circ\text{C}$ , and is analagous to hormone-receptor interactions (for review, see reference 157)



where  $[R]$ ,  $[L]$  and  $[R-L]$  represent the concentrations of receptor, lipoprotein and receptor-bound lipoprotein, respectively.  $K_D$  is the ratio of the dissociation to the association constants (ie.  $k_{-1}/k_1$ ). Since internalization of LDL at  $37^\circ\text{C}$  occurs rapidly, binding equilibrium is not achieved at this temperature, thus true  $K_D$  at  $37^\circ\text{C}$  cannot be obtained using intact cells.

Two linearization techniques were used to calculate the apparent equilibrium dissociation constant,  $K_D$ , and the maximum binding capacity,  $B_{max}$ , of LDL receptor activity. An important assumption is made in these methods of analysis

- namely, that the R-L interaction is a simple bimolecular reaction, as expressed in the equation above. In addition, the off-rate of receptor-bound ligand at 4°C is extremely slow relative to the time taken to effectively "wash" the cells from unbound ligand (83, 185). Thus, although unbound ligand (free) is removed during the washing process, the effective equilibrium binding is not disturbed.

Linearization techniques used were:

(1) A plot of the Michaelis-Menten equation (193).

Free lipoprotein (S,  $\mu\text{g}$  LDL protein/ml) was plotted against the ratio of free to receptor-bound lipoprotein (V, ng LDL protein/mg cell protein).

$B_{\text{max}}$  = the slope of the resultant straight line.

$-K_D$  = the point of intersection with the y-axis.

This technique is weighted for  $B_{\text{max}}$  analysis.

(2) Scatchard analysis (203)

The ratio of receptor-bound to free lipoprotein (V/S) was plotted against receptor-bound lipoprotein (V).

$-1/K_D$  = the slope of the resultant straight line.

$B_{\text{max}}$  = the x-intercept.

This technique is weighted for  $K_D$  analysis.

## 2.3 Results and Discussion.

### 2.3.1 Time course (4°C) of $^{125}\text{I}$ -LDL binding.

The kinetics of high-affinity  $^{125}\text{I}$ -LDL binding at 4°C were assayed on fully upregulated normal lymphoblastoid cells, as described in the legend to Fig. 2.5. The radioactivity obtained represents cell-associated  $^{125}\text{I}$ -LDL, which is equivalent to surface-bound  $^{125}\text{I}$ -LDL in this case (binding was performed at 4°C, and no ligand internalization occurs at this temperature). From the data (Fig. 2.5), it can be seen that binding reached equilibrium by 60 mins; this is similar to results obtained in cultured fibroblasts at 4°C (83).

### 2.3.2 Concentration curve (4°C) of $^{125}\text{I}$ -LDL binding.

The concentration curve of  $^{125}\text{I}$ -LDL binding at 4°C in normal human lymphoblastoid cells was determined (Fig. 2.6). Cultures were maintained for 48 hr in medium containing LPDS, to upregulate the number of LDL receptors maximally, and the cells were subsequently incubated (2 hr, 4°C) with  $^{125}\text{I}$ -LDL (0.5-20  $\mu\text{g}/\text{ml}$ ), in the absence or presence of 300  $\mu\text{g}/\text{ml}$  unlabeled LDL. (In other experiments,  $^{125}\text{I}$ -LDL concentrations of up to 300  $\mu\text{g}/\text{ml}$  were used, and similar results were obtained). Subsequent to binding and washing, each monolayer was solubilized in NaOH; the radioactivity obtained represents cell-associated  $^{125}\text{I}$ -LDL (as discussed in section 2.3.1). A two-component curve was obtained when

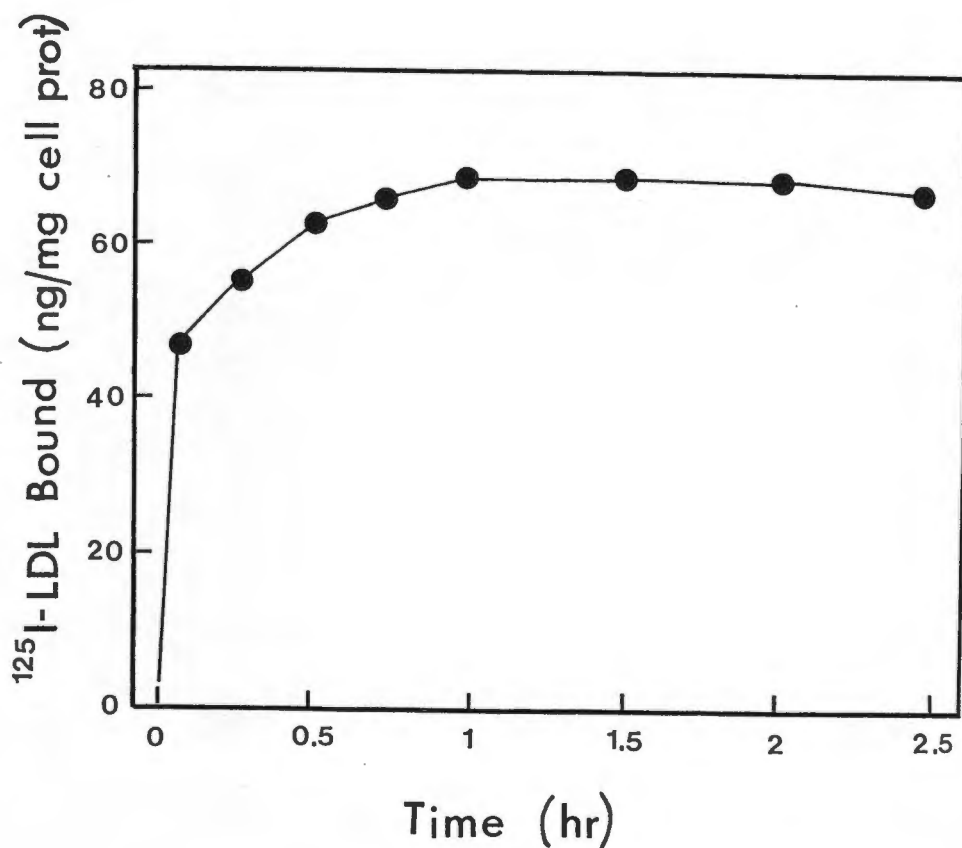


Fig. 2.5: Time course of <sup>125</sup>I-LDL binding in normal lymphoblastoid cells at 4°C.

LDL receptors on the cells were upregulated maximally by incubating the cultures in medium containing LPDS for 48 hr. The cells were then washed with PBS, and incubated at 4°C in binding medium containing 10 μg/ml <sup>125</sup>I-LDL in the absence or presence of 300 μg/ml LDL. After the indicated binding times, the high-affinity cell-associated <sup>125</sup>I-LDL was calculated as described in the "Experimental Procedures". The data are representative of results obtained in 2 separate experiments. Each point was performed in duplicate; duplicates differed by less than 3% from the means. The average content of cellular protein was 0.09 mg/10<sup>6</sup> cells.

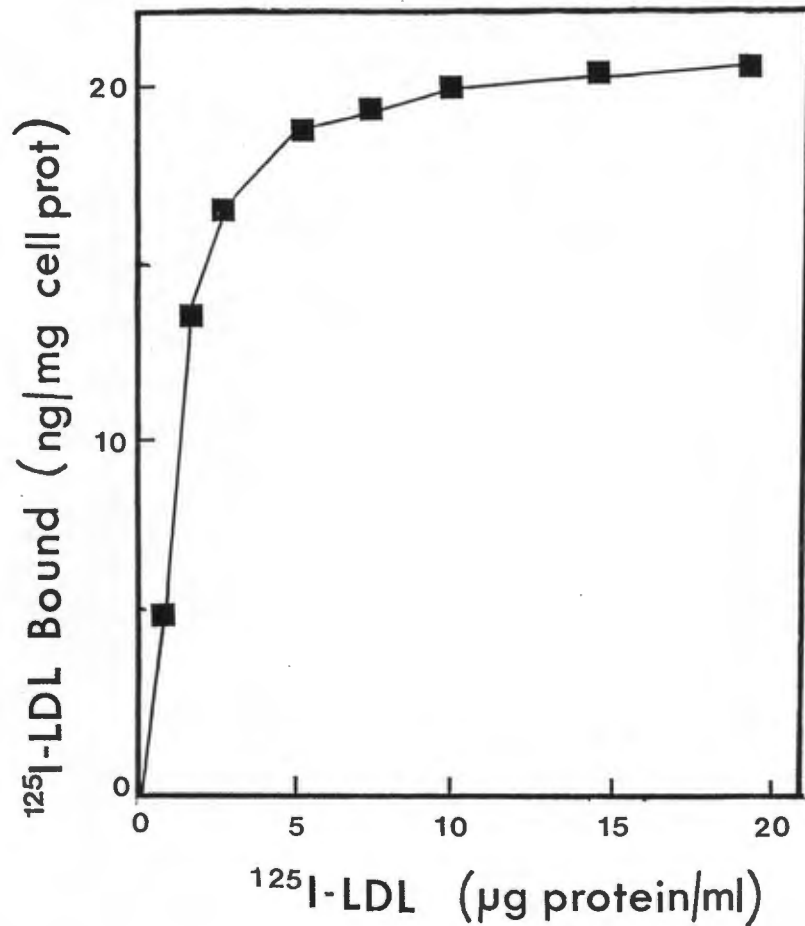


Fig. 2.6: Concentration curve of  $^{125}\text{I-LDL}$  binding at  $4^\circ\text{C}$  in normal lymphoblastoid cells.

After upregulation of the LDL receptors for 48 hr in medium supplemented with LPDS,  $^{125}\text{I-LDL}$  (at the indicated concentrations), in the absence or presence of  $300 \mu\text{g/ml}$  unlabeled LDL, was added to the cells. Binding was performed for 2 hr at  $4^\circ\text{C}$ , after which the high-affinity cell-associated ligand was calculated as described in the "Experimental Procedures". The data represent one of 2 similar experiments, performed separately. Incubations were done in duplicate; duplicates never differed by more than 6% from the mean values. The average content of cellular protein was  $0.07 \text{ mg}/10^6$  cells.

the total cell-associated  $^{125}\text{I}$ -LDL was plotted (data not shown) - such a curve typifies an apparent high-affinity, saturable LDL receptor, and a lower affinity, non-saturated component. The amounts contributed by the low-affinity processes were then subtracted from the total values. Low-affinity contribution was assessed using the lines defined either by (i) the values obtained after incubation with  $^{125}\text{I}$ -LDL in the presence of excess LDL, or (ii) an estimation of the non-specific component from the slopes of the linear component of the experimental saturation curves at the high  $^{125}\text{I}$ -LDL concentrations (applicable only in those experiments where  $^{125}\text{I}$ -LDL concentrations used were  $> 50 \mu\text{g/ml}$ ). (i) and (ii) yielded similar results. A plot of the high-affinity values is depicted in Fig. 2.6. The  $K_D$  value was  $2 \mu\text{g } ^{125}\text{I}\text{-LDL/ml}$ , and the  $B_{\text{max}}$  was about  $20 \text{ ng } ^{125}\text{I}\text{-LDL/mg cell protein}$  (see section 2.2.9 for methods of analysis). The  $K_D$  for binding at  $4^\circ\text{C}$  in HSF is 1 to  $2 \mu\text{g/ml}$  (29, 83).

Using the data obtained in the experiment depicted in Fig. 2.6, the number of LDL binding sites per lymphoblastoid cell at  $4^\circ\text{C}$  was calculated. On average, there were 1 800 binding sites per lymphoblastoid cell at  $4^\circ\text{C}$ . This value is in agreement with that reported by Ho et al (103) using similar cells, and is substantially lower than the 15 000 receptor sites detected analogously per fibroblast cell at  $4^\circ\text{C}$  (87).

### 2.3.3 Time course (37°C) of $^{125}\text{I}$ -LDL metabolism.

The surface-bound, intracellular content and degradation of  $^{125}\text{I}$ -LDL were assayed after incubating normal lymphoblastoid cells for various times in the presence of radiolabeled ligand. Prior to initiating the experiment, maximal LDL receptor expression was induced by maintaining the cells in LPDS-containing medium for 48 hours. Binding incubations were performed at 37°C with 10  $\mu\text{g/ml}$   $^{125}\text{I}$ -LDL, in the absence or presence of 300  $\mu\text{g/ml}$  unlabeled LDL, for times ranging from 10 minutes to 4 hours. The high-affinity values obtained, are depicted in Fig. 2.7. Ligand binding appears to reach a constant value between 30 and 60 mins (at 30 mins, binding is 85% of its maximum value); the intracellular content of  $^{125}\text{I}$ -LDL plateaued by 2 hours, while radiolabeled LDL degradation products were only detected in the medium at times longer than 30 mins, and increased linearly thereafter during the four hour period examined. Ho et al (103) obtained very similar results also using long-term lymphoid cells. This behaviour is similar in qualitative terms to that which has been reported for HSF: binding plateaued by 1 hour (84, 89), and detection of degradation products in the medium was possible after 30 mins (15, 84, 89). [Note: the amount of  $^{125}\text{I}$ -LDL degradation products that was detected after the standard 4 hr binding incubation performed with lymphoblastoid cells at 37°C, varied considerably from one experiment to another, using the same cell line. In all experiments, the cells were > 90% viable (as determined by trypan-blue dye exclusion), were visibly healthy (as observed under phase-

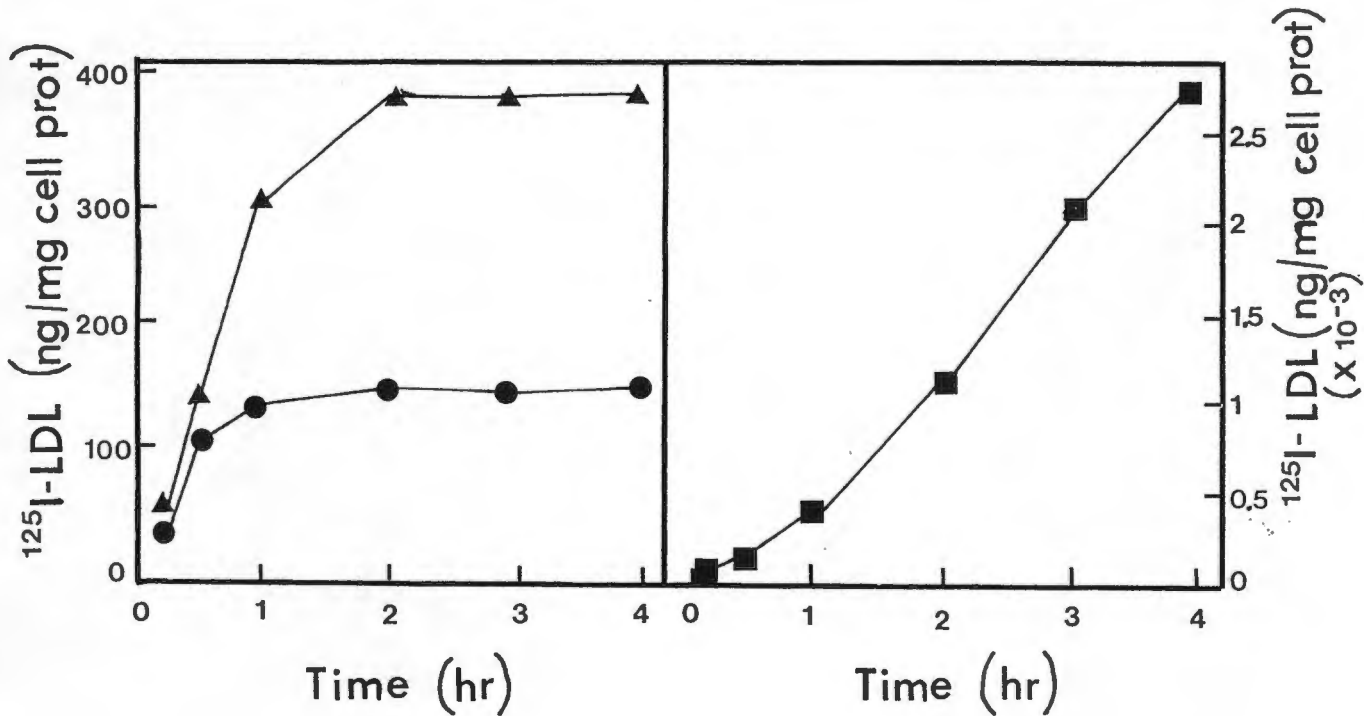


Fig. 2.7: Time course of  $^{125}\text{I}$ -LDL metabolism in normal lymphoblastoid cells, at  $37^\circ\text{C}$ .

The cells were incubated in upregulation medium for 48 hr before initiating binding incubations containing  $10\ \mu\text{g/ml}$   $^{125}\text{I}$ -LDL in the absence or presence of  $300\ \mu\text{g/ml}$  unlabeled LDL. After incubating for the indicated times at  $37^\circ\text{C}$ , high-affinity LDL which was bound (heparin releasable) (●), intracellular (▲) or degraded (■), was calculated as described in section 2.2.6. Each experimental point represents the mean of duplicate dishes; duplicates never differed by more than 3% from the means. The data were obtained in a single experiment, and are representative of the results obtained in 3 such experiments, performed separately. The average content of cellular protein was  $0.06\ \text{mg}/10^6$  cells.

contrast microscope optics), and dilutions obtained in the presence of excess unlabeled LDL were always > 90%].

The importance of receptor recycling, as regards cellular economy, is obvious: in the case of LDL receptors, despite lysosomal destruction of the internalized ligand, the receptors survive and may be recycled back to the plasma membrane for re-use (29). Thus one receptor may facilitate the internalization of many ligands during its life-time. Recycling accounts for the observation that fibroblasts continue to ingest LDL at a uniform rate for longer than 6 hours, even when the synthesis of new receptors is blocked by cycloheximide (29). The time taken for a single receptor recycling event may be calculated from the internalization index (I.D.) as follows:

Using data obtained at the 4 hr point in Fig. 2.7:

Bound (B) = 140 ng  $^{125}\text{I}$ -LDL/mg cell protein

Intracellular (I) = 380 ng  $^{125}\text{I}$ -LDL/mg cell protein

Degraded (D) = 2 800 ng  $^{125}\text{I}$ -LDL/mg cell protein

$$\text{I.D.} = (I + D)/B = (380 + 2\ 800)/140 = 22.7$$

This means that 22.7 recycling events have occurred in 4 hr i.e. one receptor cycle takes, on average, 10.5 mins. This calculation is, of course, only valid if the assumption is made that receptor number remained constant during the 4 hour incubation period, and, for example, no effective downregulation had occurred during the 4 hours. This point will be addressed again later (section 4.3.4). A second assumption made is that no receptors are recruited from a significant (previously inactive) pool during the four hour incubation time. This assumption has been shown to be valid

in HSF (16), but might not be the case for the lymphoblastoid cells. Recycling times of about 12 mins have been reported for this receptor in fibroblasts (15, 29, & 30).

#### 2.3.4 Concentration curves (37°C) of $^{125}\text{I}$ -LDL metabolism.

The concentration curves of  $^{125}\text{I}$ -LDL metabolism in human lymphoblastoid cells derived from an LDL receptor normal subject were determined as described in the legend to Fig. 2.8. Incubations were performed for 4 hours at 37°C with  $^{125}\text{I}$ -LDL (2-50  $\mu\text{g}/\text{ml}$ ), in the absence or presence of 300  $\mu\text{g}/\text{ml}$  unlabeled LDL. (In other experiments,  $^{125}\text{I}$ -LDL concentrations of up to 300  $\mu\text{g}/\text{ml}$  were used, and similar results were obtained). Surface-bound  $^{125}\text{I}$ -LDL was distinguished from that which was internalized by treatment of the cells with the sulfated glycosaminoglycan, heparin - an agent which removes more than 75% of the total cell-surface associated LDL (83). The high-affinity saturation curves of  $^{125}\text{I}$ -LDL bound, internalized or degraded, were obtained as described in section 2.3.2, and are depicted in Fig. 2.8.

Half-maximal activity was obtained at 12  $\mu\text{g}$   $^{125}\text{I}$ -LDL/ml and a maximum binding activity of about 90 ng  $^{125}\text{I}$ -LDL/mg cell protein was measured. Half-maximal activity of HSF was reported to be achieved at 10-15  $\mu\text{g}$  LDL protein/ml (84).

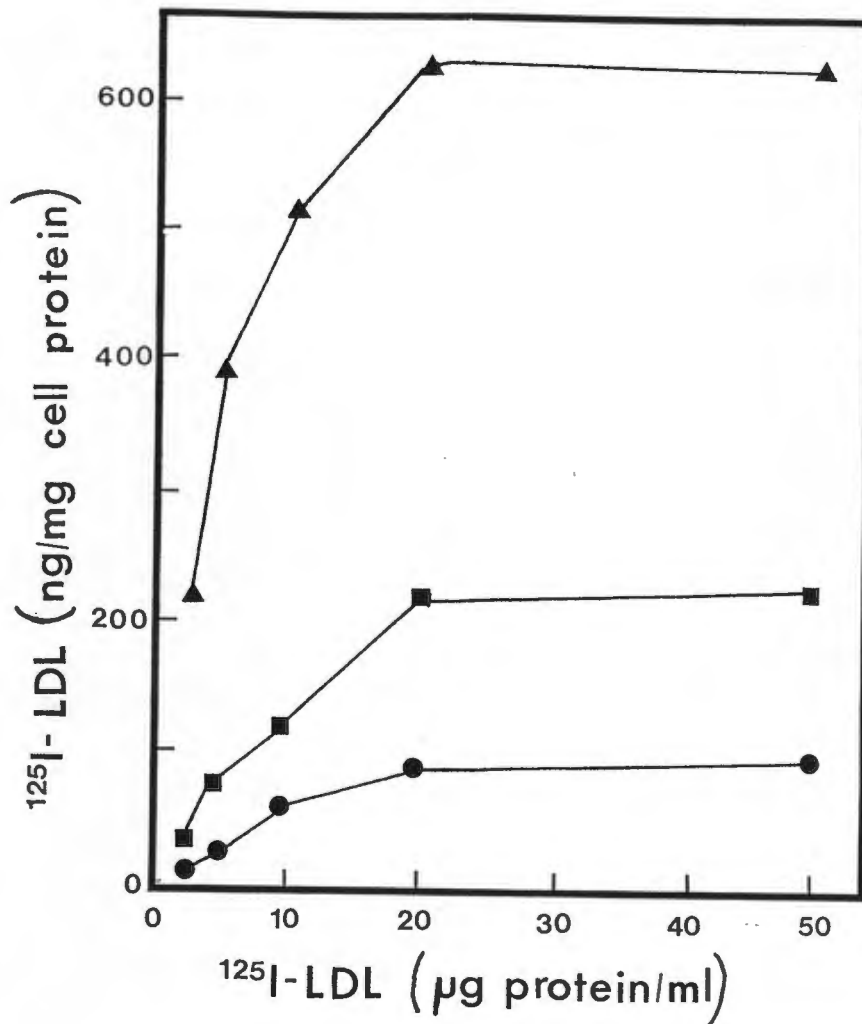


Fig 2.8: Concentration curves of  $^{125}\text{I-LDL}$  metabolism in normal human lymphoblastoid cells.

Lymphoblastoid cells were incubated for 48 hr in medium supplemented with LPDS to upregulate the number of LDL receptors maximally. The cells were then resuspended in fresh medium containing  $^{125}\text{I-LDL}$  (at the indicated concentrations) in the absence or presence of 300  $\mu\text{g/ml}$  LDL, for 4 hr at 37°C. High-affinity heparin releasable  $^{125}\text{I-LDL}$  (●), intracellular  $^{125}\text{I-LDL}$  (■) and degraded  $^{125}\text{I-LDL}$  (▲) were assayed and calculated as described in "Experimental Procedures". The data, obtained from duplicate dishes in a single experiment, is representative of 2 similar experiments, performed separately. Duplicate values never differed by more than 5% from the means. The average content of cellular protein was 0.055 mg/ $10^6$  cells.

### 2.3.5 LDL receptor binding - some general aspects.

Acetylation of LDL results in the chemical modification of a number of lysine residues in the LDL apoprotein, producing an increased net negative charge at neutral pH. Such modified (acetylated LDL) did not bind the receptor in lymphoblastoid cells, using a range of  $^{125}\text{I}$ -AcLDL from 10 to 200  $\mu\text{g/ml}$  (data not shown). In addition, the presence of excess unlabeled AcLDL (300  $\mu\text{g/ml}$ ) did not compete with  $^{125}\text{I}$ -LDL for binding sites. The inability of lymphoblastoid cells to catabolize negatively-modified LDL is common to the majority of cultured cell types, such as fibroblasts (31).

The high-affinity binding and degradation of LDL in normal lymphoblastoid cells was strictly dependent on the presence of calcium ions in the incubation medium (Table 2.1). The addition of 3 mM EGTA (a calcium ion chelating agent) to the binding medium, in the absence of any added calcium, produced a virtual loss of high-affinity  $^{125}\text{I}$ -LDL binding and degradation. This effect was reversible, since binding and metabolism were partially restored after the removal of the EGTA. It is well established that the LDL receptors in cultured fibroblasts also show an absolute requirement for divalent cations to effect ligand binding (84, 87). Goldstein et al (87) have shown that the addition of 0.1 mM EDTA prevented  $^{125}\text{I}$ -LDL binding (4°C) in HSF (calcium-free medium was used in these experiments); this inhibition was completely overcome by the presence of low concentrations of calcium or manganese ions; magnesium ions were also able to restore binding, but only at higher levels.

**Table 2.1: Binding and metabolism of  $^{125}\text{I}$ -LDL in cultured lymphoblastoid cells is calcium dependent.**

Lymphoblastoid cells were incubated for 48 hr in medium containing LPDS, to induce maximal upregulation of LDL receptors. The cells were then washed with PBS, and resuspended in media which contained either 10  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -LDL + 3 mM EGTA, in the presence or absence of 300  $\mu\text{g}/\text{ml}$  unlabeled LDL, for 4 hr at 37°C (A), or 3 mM EGTA only, for 4 hr. In the latter case, this medium was replaced with medium containing 10  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -LDL  $\pm$  300  $\mu\text{g}/\text{ml}$  LDL (in the absence of EGTA) for a subsequent 4 hr at 37°C (B).  $^{125}\text{I}$ -LDL which was heparin releasable, intracellular or degraded was assayed as described in "Experimental Procedures". The data were obtained from duplicate dishes; duplicates always differed by less than 5% from the means. Tabulated values represent results expressed as a % of the control dishes which were incubated identically, except that EGTA was always omitted.

Incubation conditions	Bound	Intra-cellular	Degraded
A + EGTA (4 hr, 37°C)	14%	<5%	<5%
B + EGTA (4 hr, 37°C), then - EGTA (4 hr, 37°C)	94%	70%	60%

### 2.3.6 The site of $^{125}\text{I}$ -LDL degradation.

The lysosome, an organelle originally characterized in biochemical terms by de Duve (56), and known to contain a large number of acid hydrolases easily capable of digesting the LDL components, was considered to be the most probable organelle where LDL could be degraded. In human fibroblasts, this proposed lysosomal digestion of LDL has been confirmed (i) using cells obtained from patients with a genetic deficiency of lysosomal acid lipase (90) and (ii) using agents such as chloroquine and leupeptin in normal cells. Chloroquine has been shown to increase the pH of acidic intracellular compartments; the increased lysosomal pH results in an inhibition of lysosomal enzymes (54). In addition, chloroquine also directly inhibits cathepsin B (257). The latter lysosomal protease is also directly inhibited by leupeptin (7, 246). Fibroblasts, incubated in the presence of chloroquine, showed a dramatic decrease in the rate of  $^{125}\text{I}$ -LDL degradation, and an almost equivalent increase in the amount of intracellular ligand (89). Furthermore, results from this laboratory (12, 249) demonstrated that the intralysosomal degradation of apolipoprotein B involves an initial limited endoproteolytic attack at susceptible sites by cathepsin D. This and other enzymes, including cathepsin B, then act synergistically to bring degradation to completion. The results illustrated in Fig. 2.9 were obtained in an experiment using chloroquine in lymphoblastoid cultures. Incubations were performed at 37°C

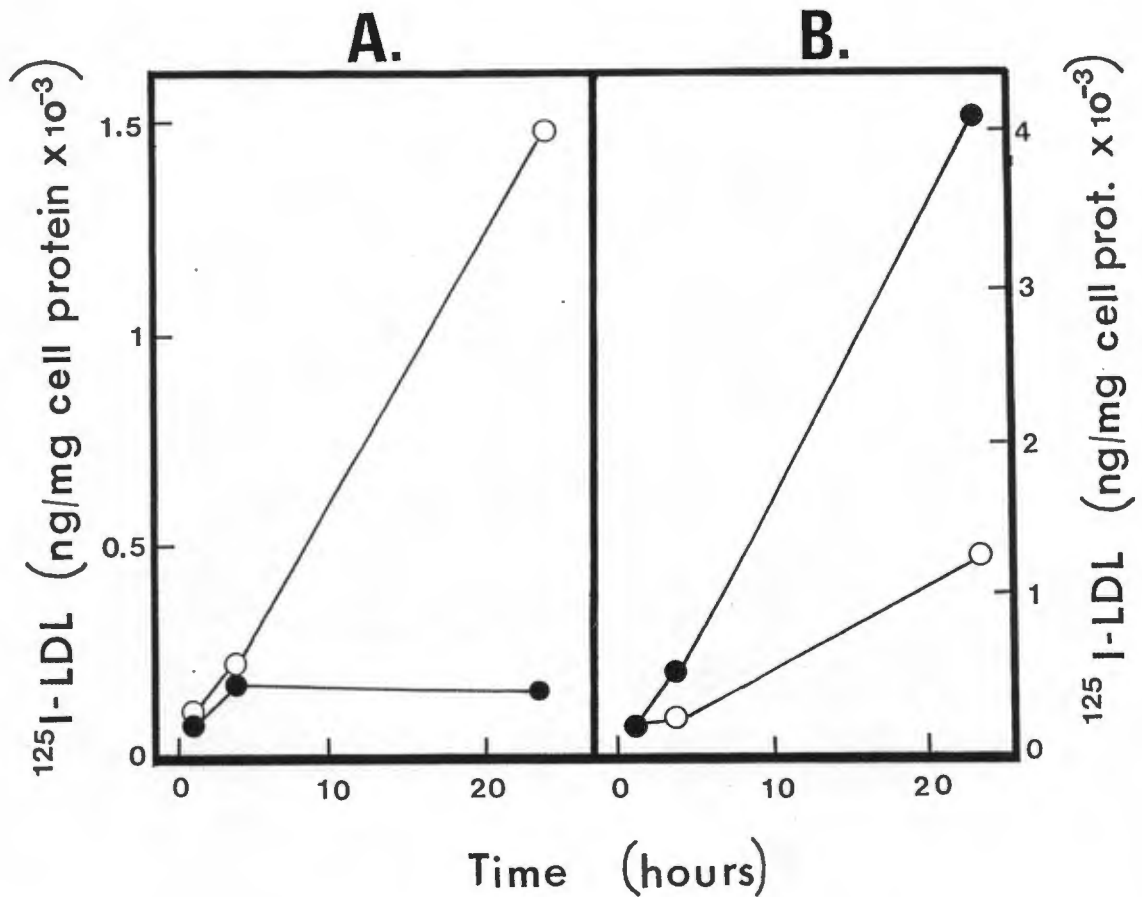


Fig. 2.9: The effect of chloroquine (60  $\mu\text{M}$ ) on the metabolism of  $^{125}\text{I}$ -LDL in normal lymphoblastoid cells. Cells were upregulated, then incubated for the indicated times at 37°C in binding medium containing 10  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -LDL in the absence or presence of 300  $\mu\text{g}/\text{ml}$  unlabeled LDL, with (O), or without (●) chloroquine. High-affinity values for  $^{125}\text{I}$ -LDL which was A: intracellular or B: degraded, were calculated as described (section 2.2.6) from duplicate dishes at each point. Duplicates always differed by < 5% from the means. These results were obtained in a single experiment, and typify those obtained in 2 separate experiments.

for times ranging from 1-24 hours in binding medium containing 10  $\mu\text{g/ml}$   $^{125}\text{I}$ -LDL, in the absence or presence of 300  $\mu\text{g/ml}$  unlabeled LDL, with or without 60  $\mu\text{M}$  chloroquine. The results show that the addition of chloroquine caused a marked inhibition in the rate of ligand degradation, accompanied by an increase in the amount of intracellular  $^{125}\text{I}$ -LDL. The reversibility of the chloroquine effect has been demonstrated in cultured fibroblasts: 7 hours after the removal of this agent, proteolysis was found to resume (89). Various investigators have reported the ability of chloroquine to block the degradation of other receptor-bound and internalized proteins (17, 37).

#### 2.3.7 $^{125}\text{I}$ -LDL metabolism in cultured lymphoblastoid and fibroblast lines, obtained from 4 different FH patients.

The high-affinity binding, intracellular content and degradation of  $^{125}\text{I}$ -LDL in cultured human lymphoblastoid cells was measured in cell lines derived from 2 unrelated FH heterozygous patients and 2 unrelated FH homozygous patients. These values were compared with data obtained by other workers in this laboratory using HSF from the same 4 FH patients (248). The results, which are listed in Table 2.2, clearly show that similar trends are found in fibroblasts and lymphoblastoid cells derived from these patients. Cells which are heterozygous for the LDL receptor show a reduction (of about 30-50%) in the binding, internalization and degradation of labeled ligand, relative to normal subjects; the cells derived from homozygous

Table 2.2:  $^{125}\text{I}$ -LDL metabolism in cultured lymphoblastoid cells (L), derived from 4 different FH patients, and a comparison with  $^{125}\text{I}$ -LDL metabolism in the corresponding fibroblasts (F).

Lymphoblastoid cells, derived from 2 different FH heterozygous patients and 2 different FH homozygous patients, were first incubated in LPDS containing medium (section 2.2.5). The cells were then maintained for 4 hr at 37°C in medium containing LPDS, supplemented with 10  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -LDL in the absence or presence of 300  $\mu\text{g}/\text{ml}$  unlabeled LDL. High-affinity values for  $^{125}\text{I}$ -LDL which was bound (heparin releasable), internalized or degraded, were calculated from duplicate dishes; duplicates never differed by more than 5% from the means. In the case of the lymphoblastoid cell lines, the results shown were obtained in a single experiment which is representative of 3 similar experiments. The data for the HSF were obtained by other workers in this laboratory (Table 2, ref. 248). All results are expressed as a % of the values obtained from normal lymphoblastoid or HSF cell lines.

LDL receptor status of subject	$^{125}\text{I}$ -LDL (% of normal value)					
	Bound		Intracellular		Degraded	
	L	F	L	F	L	F
Heterozygous	40	64	62	73	73	88
Heterozygous	66	59	63	72	71	84
Homozygous defective	3	7	7	5	5	6
Homozygous defective	12	14	10	20	18	24

defective patients exhibited values that ranged from 3-25% of normal. These studies, and those done by others (103, 248) prove that normal lymphoblastoid cells possess LDL receptors, and that such receptors are deficient in FH homozygote and heterozygote patients. The decreased receptor number results ultimately in diminished ligand degradation, since endocytosis, (which is receptor-mediated), is normally responsible for most of the ligand degradation. Kayden et al (121) found that long-term lymphoid cells from FH homozygote patients showed no suppression of HMG CoA reductase activity, or stimulation of cholesteryl ester formation, when incubated with LDL. This provides additional evidence for the lack of LDL receptors on these cells in such patients.

#### 2.3.8 The degradation of scrape-loaded $^{125}I$ -LDL - a comparative perspective.

We used the technique of scrape-loading, originally described by McNeil et al (161), as a means to introduce  $^{125}I$ -LDL into adherant cultured cells via non-receptor-mediated processes. Essentially, fibroblasts were scraped with a rubber policeman in the presence of medium containing the desired radiolabeled macromolecule, in order to effect incorporation independently of the receptors. The cells were subsequently replated and incubated in a 37°C humidified incubator; at various times, the metabolism of the incorporated ligand was assayed as described (section 2.2.8). Scrape-loading experiments were always performed

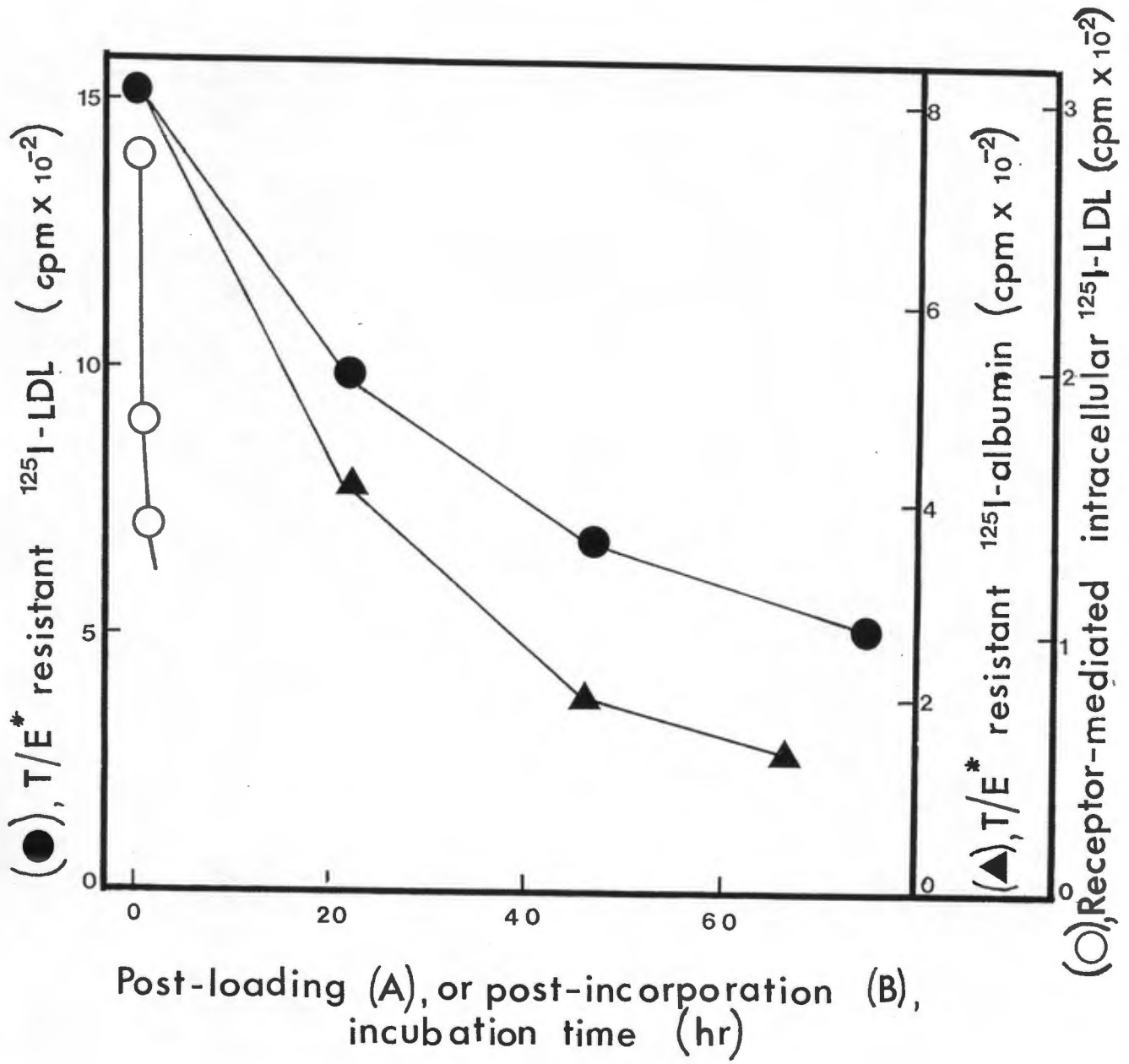
with the LDL receptor negative human skin fibroblast line, LVS.

$^{125}\text{I}$ -LDL which was scrape-loaded into the LDL receptor negative fibroblasts, disappeared from the intracellular trypsin/EDTA-resistant compartment with first order kinetics; the half-life of this disappearance was about 50 hours (Fig. 2.10). Folch extractions (71) performed concomitantly confirmed that very little radioactivity was lipid-associated (< 1%).

Control experiments were performed (Fig. 2.10) in which the labeled macromolecule loaded was  $^{125}\text{I}$ -albumin (iodinated by the Iodogen method, essentially as described in section 3.3.2, using a 20 mg/ml BSA solution containing 50 mM NaCl and 10 mM sodium phosphate, pH 7.4; specific activities obtained were generally about 100-200 cpm/ng protein). The  $^{125}\text{I}$ -BSA disappeared from the trypsin/EDTA resistant pool with a half-life of about 20 hours. This was consistent with results reported by others (62, 63): these investigators have shown that BSA, after scrape-loading into 3T3-L1 cells, is found in the cytosolic compartment, and is degraded with a half-life of about 20 hours.

The value of 50 hr for  $^{125}\text{I}$ -LDL disappearance - after incorporation into HSF by scrape-loading - was compared with the half-life of the same ligand after uptake by intact cells via receptor-mediated endocytosis. To this end, the LDL receptor normal fibroblast line GM 3348A, was seeded into petri dishes, grown and incubated in DMEM/LPDS for

FIGURE 2.10 (legend on next page)



\* Trypsin/EDTA

Fig. 2.10:  $^{125}\text{I}$ -ligand metabolism subsequent to incorporation into LDL receptor negative fibroblasts by scrape loading ( $\bullet, \blacktriangle$ ) (A), or  $^{125}\text{I}$ -LDL metabolism subsequent to uptake via receptor mediated endocytosis in normal fibroblasts ( $\circ$ ) (B).

A:  $^{125}\text{I}$ -LDL (200  $\mu\text{g}/\text{ml}$ ) ( $\bullet$ ) or  $^{125}\text{I}$ -Albumin (200  $\mu\text{g}/\text{ml}$ ) ( $\blacktriangle$ ) were incorporated into separate sets of LDL receptor negative fibroblasts (LV5), as described in "Experimental Procedures" (section 2.2.8), and in the text (section 2.3.8). After incubating in DMEM/LPDS for various times, the amounts of trypsin/EDTA resistant radioactivity were determined. Results are representative, in each case, of 3 separate experiments; data are the means of duplicates, which never differed from the mean values by more than 6%.

B: Monolayers of LDL receptor normal fibroblasts were prepared as described in the text. Pulse-label incubations were performed in DMEM/LPDS supplemented with 10  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -LDL  $\pm$  200  $\mu\text{g}/\text{ml}$  of unlabeled LDL for 3 hours. After removal of the medium, the monolayers were thoroughly chilled, washed four times with PBS-ALB and three times with PBS, at 4°C. Ice-cold sodium heparin solution (0.4% in PBS) was added to each dish of cells for 1 hour at 4°C, after which the monolayers were washed four times with PBS (4°C). Chase incubations were then carried out on the intact cell layers at 37°C in DMEM/LPDS for various times. The data plotted are high-affinity values, calculated as described in section 2.2.6, and were obtained from duplicate dishes; duplicates did not differ by more than 7% from the means.

48 hr to upregulate LDL receptors maximally (section 2.2.5). The cells were then pulse-labeled at 37°C with medium containing 10 µg/ml <sup>125</sup>I-LDL in the absence or presence of 200 µg/ml unlabeled LDL, to measure receptor-mediated ligand uptake. After 3 hours, this medium was removed, and the monolayers were treated with heparin at 4°C to remove all surface receptor-bound <sup>125</sup>I-LDL. The cells were then chase-incubated at 37°C for various times, and the metabolism of this intracellular <sup>125</sup>I-LDL pool was analysed as described in the scrape-loading procedure (section 2.2.8). The results, which are depicted in Fig. 2.10, indicate that about 50% of the ligand is degraded within one hour. These data are consistent with the well-documented time course of <sup>125</sup>I-LDL processing in cultured fibroblasts: in normal fibroblasts, the receptor-bound LDL remains on the surface for less than 10 mins, on average; within this time, most of the surface-bound LDL particles enter the cell, and within 30-60 mins, the protein component of <sup>125</sup>I-LDL is digested to amino acids and the <sup>125</sup>I, which had been attached to the tyrosine residues on LDL, is released into the culture medium as <sup>125</sup>I-monoiodotyrosine (83, 84).

The compartmental distribution of scrape-loaded, radioactive ligand was analysed at various post-incorporation times (Fig. 2.11). Total radioactivity within the system remained constant, while the distribution of this label within the various cellular compartments varied with time. This is consistent with an intact system in which the metabolism of the incorporated ligand occurs. The amount of material that was trypsin/EDTA resistant decreased with time

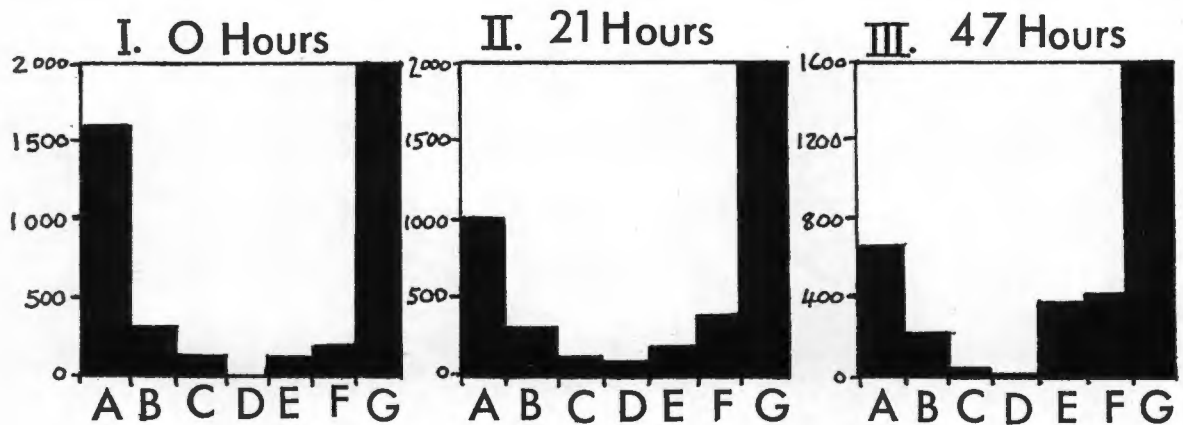


Fig. 2.11: Compartmental analysis of  $^{125}\text{I}$ -LDL, subsequent to incorporation in fibroblasts by scrape-loading.

$^{125}\text{I}$ -LDL was scrape-loaded into confluent LDL receptor negative fibroblasts as described in Section 2.2.8. After post-label incubating for 0 hours (I), 21 hours (II) or 47 hours (III), compartmental analyses were performed.

- A. Trypsin/EDTA resistant material.
- B. Trypsin/EDTA releasable material.
- C. PBS wash number 1.
- D. PBS wash number 2.
- E.  $^{125}\text{I}$ -LDL degradation products in the medium.
- F. Radioactivity in TCA pellet (ie. a count of the TCA pellet obtained in E - see section 2.2.8 (i)).
- G. Total counts.

Similar results were obtained in 3 separate experiments.

The data were derived from duplicate assays of each compartment, at each time.

(Fig. 2.11, column A, and also Fig. 2.10A); this was balanced by an increase in  $^{125}\text{I}$ -LDL degradation products detected in the medium, and an increased amount of radioactivity measured in the TCA pellets (Fig. 2.11, columns E and F, respectively). Thus, although some of the  $^{125}\text{I}$ -LDL was undoubtedly degraded during the chase incubation, some relatively intact (TCA precipitable) material was released from the cells.

#### 2.3.9 Concluding remarks.

In this study, the binding and metabolism of  $^{125}\text{I}$ -LDL were examined in cultured human lymphoblastoid cells, after ligand uptake via receptor-mediated endocytosis, and the results were compared with those documented for human skin fibroblasts. In Table 2.3, this comparative study is summarized. Both cell types required calcium ions for ligand binding. The simultaneous addition of excess unlabeled LDL (200-300  $\mu\text{g}/\text{ml}$ ) during binding ( $4^\circ\text{C}$  or  $37^\circ\text{C}$ ) produced a > 90% decrease in the amount of radiolabeled ligand bound. Furthermore, modified (negatively charged) acetylated LDL was unable to bind to the LDL receptor. The competition obtained in the presence of excess unlabeled ligand, and the lack of binding of modified ligand, are indicative of the specificity of the LDL receptor for LDL. The number of LDL receptors on the cell surface of the two culture types under discussion differed greatly: there were 1 800 such receptors per lymphoblastoid cell, compared with 15 000 on each fibroblast cell.  $K_D$  values for binding were

**Table 2.3: A comparison of  $^{125}\text{I}$ -LDL binding and metabolism in cultured human lymphoblastoid and fibroblast cell lines.**

Parameter	L <sup>1</sup>	F <sup>2</sup>	Ref <sup>3</sup>
Ca <sup>++</sup> dependence of binding	yes	yes	84, 87
Binding of AcLDL	no	no	31
K <sub>D</sub> ( $\mu\text{g/ml}$ )(4°C binding)	2	1-2	29
Time for binding to plateau at 4°C (mins).	30-60	30-60	83
Time for degradation products to appear at 37°C (mins)	>30	>30	15, 84
Effect of chloroquine on LDL degradation.	decr	decr	89
Receptor recycling time (mins)	10.5	12	29,30
No. of LDL receptors on cell surface (4°C).	1 800	15 000	87
$^{125}\text{I}$ -LDL binding in:			
FH heterozygotes (% of normal)	50-70	50-70	248
FH homozygotes (% of normal)	3-25	3-25	248

1 = Lymphoblastoid cells  
 2 = Human skin fibroblasts  
 3 = Reference(s) for HSF data

similar in both cell types, viz. about 2  $\mu\text{g}$   $^{125}\text{I}$ -LDL/ml (4°C). When incubated at 37°C, LDL receptors were internalized and were subsequently recycled back to the cell surface within about 10 minutes;  $^{125}\text{I}$ -LDL degradation products were detected in the incubation media after 30 minutes. The addition of the lysosomotropic agent, chloroquine, resulted in an inhibition of the degradation process. The critical importance of surface LDL receptors - the initial component of the receptor-mediated endocytotic pathway - was emphasised by the finding that lymphoblastoid and fibroblast cells derived from patients with the receptor negative form of homozygous FH, which lack functional LDL receptors, were unable to take up LDL with high affinity.

Subsequent to endocytosis via receptor-mediated processes, it is known that the LDL receptor and its ligand, LDL, dissociate in the acidic compartment of uncoupling of receptor and ligand (CURL) (77). It is widely accepted that the majority of the LDL receptors are recycled back to the cell surface (77), while a few are degraded; the processes that determine at what stage a receptor may be routed into the latter pathway, and precisely how this degradation is affected, have not been elucidated to date (see Section Four). In contrast, the degradation of LDL, subsequent to uptake via receptor-mediated endocytosis, has been well characterized as a lysosomal event (Table 2.3, and references 34, 89).

The time taken to degrade LDL, which had been endocytosed in normal fibroblasts via LDL receptors, was examined: it was

found that about 50% of this ligand was degraded within one hour (Fig. 2.10). For comparative purposes,  $^{125}\text{I}$ -LDL was introduced into the cytosol of cultured fibroblasts by scrape-loading (61), and the metabolism of the ligand was then analysed: about 50% of the  $^{125}\text{I}$ -LDL was degraded by 50 hours, which was markedly slower than the degradation rate observed for ligand which had been receptor endocytosed. This result gives some perspective as to the relative rapidity of receptor-mediated delivery of ligand to lysosomes.

Mayer et al (159) have proposed a general eukaryotic protein turnover cycle, based on protein synthesis and degradation studies in several animal models, which involves translocation ie. proteins enter functioning organelles after synthesis, and subsequently leave after degradation (those proteins possessing properties precluding such transfer - such as mitochondrial inner membrane proteins - would be excluded from this process). Kinetic considerations were later incorporated into the proposed cycle (158), to yield a model in which proteins would be translocated from functional sites at heterogeneous rates for degradation (by lysosomal or non-lysosomal mechanisms). Measured protein half-lives would, presumably, reflect the rate of the translocative events.

In order to investigate such protein translocation, Mayer and co-workers have made extensive use of the macromolecule loading techniques (outlined in section 1.1). Such methodology is free of the complications inherent in the

classically used pulse-chase labeling procedures for analysis of protein catabolism (eg. the radiolabeling of all cellular proteins during the pulse period), and has the advantage that labeled, soluble macromolecules may be loaded into selected target cells.

Evidence has recently been provided by Doherty et al (63) that the microinjection of various glycolytic enzymes into 3T3-L1 cells results in their rapid sequestration to the nuclear-intermediate filament fraction (a cell fraction rich in vimentin and histones), followed by their subsequent slow delivery to the lysosomal system. It is likely that a constitutive autophagic process is involved in this delivery. Results obtained by Earl et al (68, 69) provide additional support for this mechanism. These researchers used reconstituted Sendai-viral envelopes to implant viral HN and F proteins into the surface of cultured hepatoma cells, as a means to investigate the mechanism(s) of plasma membrane protein catabolism. They were able to show that the transplanted HN and F proteins were sequestered into a perinuclear site, tightly associated with nuclear-intermediate-filament material, and were then progressively transferred from this fraction into the autophagolysosomal system for degradation.

In an attempt to define the molecular criteria that target proteins into intracellular degradative pathways, Gaskell et al (75) microinjected [<sup>35</sup>S]polypeptides (ie. precursor polypeptides lacking co- and post-translational modifications) into erythrocytes or 3T3-L1 cells. Very little

**SECTION THREE****OPTIMIZATION OF METHODS TO DETERMINE THE HALF-LIFE OF THE  
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### 3.1 Introduction.

It is widely known that certain cell surface receptors play an essential role in cellular homeostasis by mediating the endocytosis of biologically important molecules, such as nutrients eg. LDL (88), hormones eg. insulin (154) and growth factors eg. EGF (65). Subsequent to internalization, the fates of the ligands and their receptors are different in various cases: ligands may be transported across the cell, stored intracellularly or degraded, while receptors may be recycled (with or without the endocytosed ligand) for re-use, or degraded. The rates at which receptors are turned over (ie. their half-lives) may play a significant role in determining how quickly, and to what extent, any given cell will be able to respond to alterations in its external environment.

Turnover rates of a number of receptors have been determined in different cell types, using a variety of methods: it appears that most mammalian plasma membrane proteins have half-lives of between 10 and 80 hours eg. the half-life of the EGF receptor in a human epidermoid carcinoma cell line is 20 hours, in the absence of ligand (55); the asialoglycoprotein receptor in Hep G2 cells turns over with a half-life of 30 hours (216); the transferrin receptor in a human leukemic T-cell line has a half-life of 60 hours (173), and the upregulated insulin receptor in cultured human lymphocytes, one of about 11 hours (120). In comparison, the half-life of the LDL receptor in human skin fibroblasts was reported in 1975 by Brown and Goldstein (32)

to be about 25 hours, as measured by the decline of surface receptor activity after the administration of the protein synthesis inhibitor, cycloheximide. (The proposed effect of this agent on the receptor half-life will be addressed in section 4.3.5 and 4.4).

In this section, the development and optimization of the [<sup>35</sup>S]-methionine pulse-chase procedure is detailed, as well as the obtaining and characterization of IgG-C7 (the monoclonal antibody to the ligand binding domain of the LDL receptor). Essentially, the pulse-chase method involved biosynthetically labeling intact cells with radioactive methionine, followed by a subsequent immunoprecipitation of LDL receptors using IgG-C7. Quantitation of the fluorograms obtained from electrophoretically separated [<sup>35</sup>S]-labeled proteins provided a definitive means for assessing the turnover rate of the LDL receptors. The major advantage of this technique is that cells can be biosynthetically labeled with a radioactive, essential amino acid, and the receptor followed in a benign manner, without the use of inhibitors which often introduce artifacts due to impairment of cellular function. In addition, the turnover rate of LDL receptors was compared with that of another cell surface receptor (namely, the transferrin receptor). To this end, a monoclonal antibody to the latter receptor was used to immunoprecipitate the transferrin receptors from the very same cell extracts from which the LDL receptors were precipitated.

### 3.2 Monoclonal antibody to the LDL receptor (IgG-C7).

#### 3.2.1 IgG-C7 producing hybridoma cells.

A hybridoma cell line which produced IgG-C7 was purchased from the American Type Culture Collection (Rockville, Maryland, USA, CRL 1691). The general procedure for obtaining such a cell line is as detailed by Beisiegel *et al* (23). In brief, LDL receptors were isolated from bovine adrenal cortex, solubilized with octylglucoside and partially purified by DEAE-cellulose and agarose gel column chromatography (211). Mice were immunized with the partially purified receptor protein, and the spleen cells were fused with mouse myeloma cells using polyethylene glycol, according to standard methods (23). Medium from the fused cells was assayed for anti-LDL receptor activity, and the hybridoma cells that were positive were cloned by limiting dilution (23).

#### **General aspects.**

The commercially obtained hybridoma cell line was grown in suspension in 90% DMEM with 4.5 g/l glucose, supplemented with 10% HIFCS. Cells were routinely grown in sterile 90 mm petri dishes, each containing 10 ml medium with hybridoma cells at approximately  $0.3 \times 10^6$  cells/ml. Typically, healthy cells adhered to the bottom of the dishes, but these were easily dispersed with gentle aspiration. Medium was changed on growing cultures every alternate day. To grow cultures for ascites production, the cells were aspirated, and

aliquoted into 4 separate 90 mm petri dishes, and the volume of medium in each dish was made up to 10 ml. After 2-3 days, the procedure was repeated, until the required number of dishes was obtained. Periodically, actively growing cell cultures were centrifuged (2 000 rpm, 2 mins, room temp) and resuspended in 90% HIFCS and 10% dimethylsulphoxide (at  $2 \times 10^6$  cells/ml). The cells were aliquoted into nunc vials (1 ml/vial) and frozen in the gaseous phase of liquid nitrogen, prior to being stored under liquid nitrogen (164). Thawing of each vial was done rapidly at 37°C, and the cells were washed immediately with medium (10 ml) by centrifugation before being seeded into 90 mm petri dishes.

#### Growth characteristics.

The IgG-C7 producing hybridoma cells typically displayed a logarithmic growth pattern (Fig. 3.1, Curve A) for about 48 hours, with a doubling time of 15 hours, after which the rate of cell growth declined. This decline was due at least in part to medium depletion since, if the medium was changed at regular intervals during cell growth, the logarithmic growth pattern was maintained longer (about 72 hours), while the maximum cell density that was attained increased substantially (Fig. 3.1, Curve B).

#### A control hybridoma cell line.

A mouse hybridoma cell line which produced antibodies directed against an irrelevant antigen (testosterone) was obtained as a gift from Bioclones (Stellenbosch, S.A.). The cells were grown in medium containing RPMI, supplemented with 10% HIFCS, 300 mg/l L-glutamine, 500  $\mu$ M mercapto-

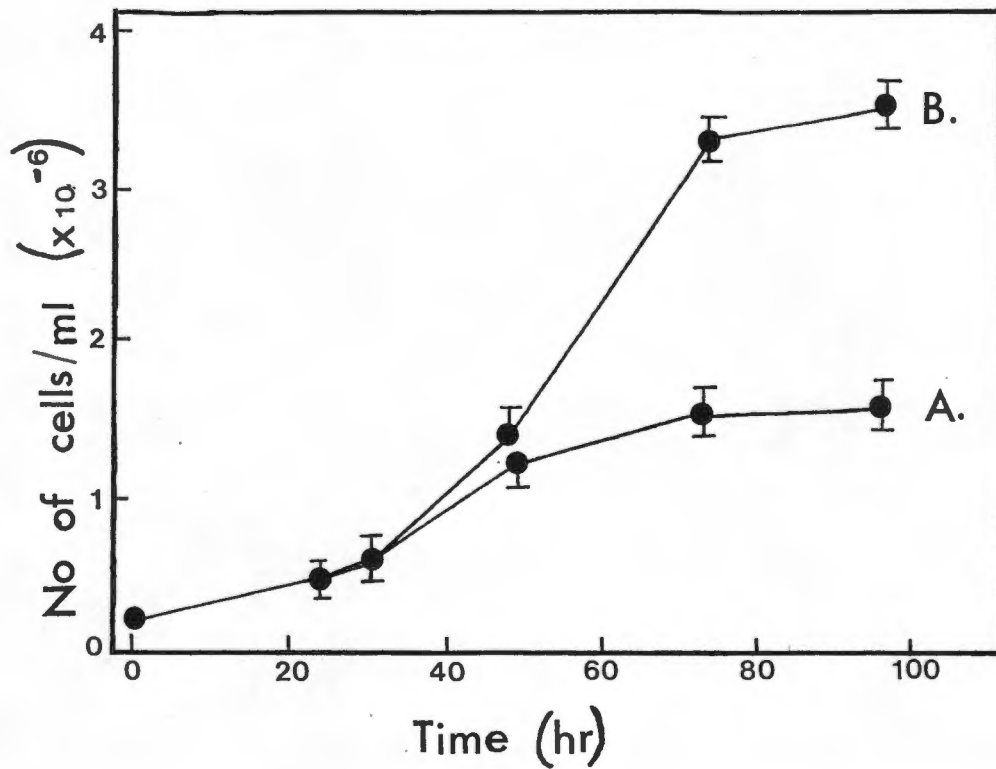


Fig. 3.1: Growth curves of IgG-C7 producing hybridoma cells.

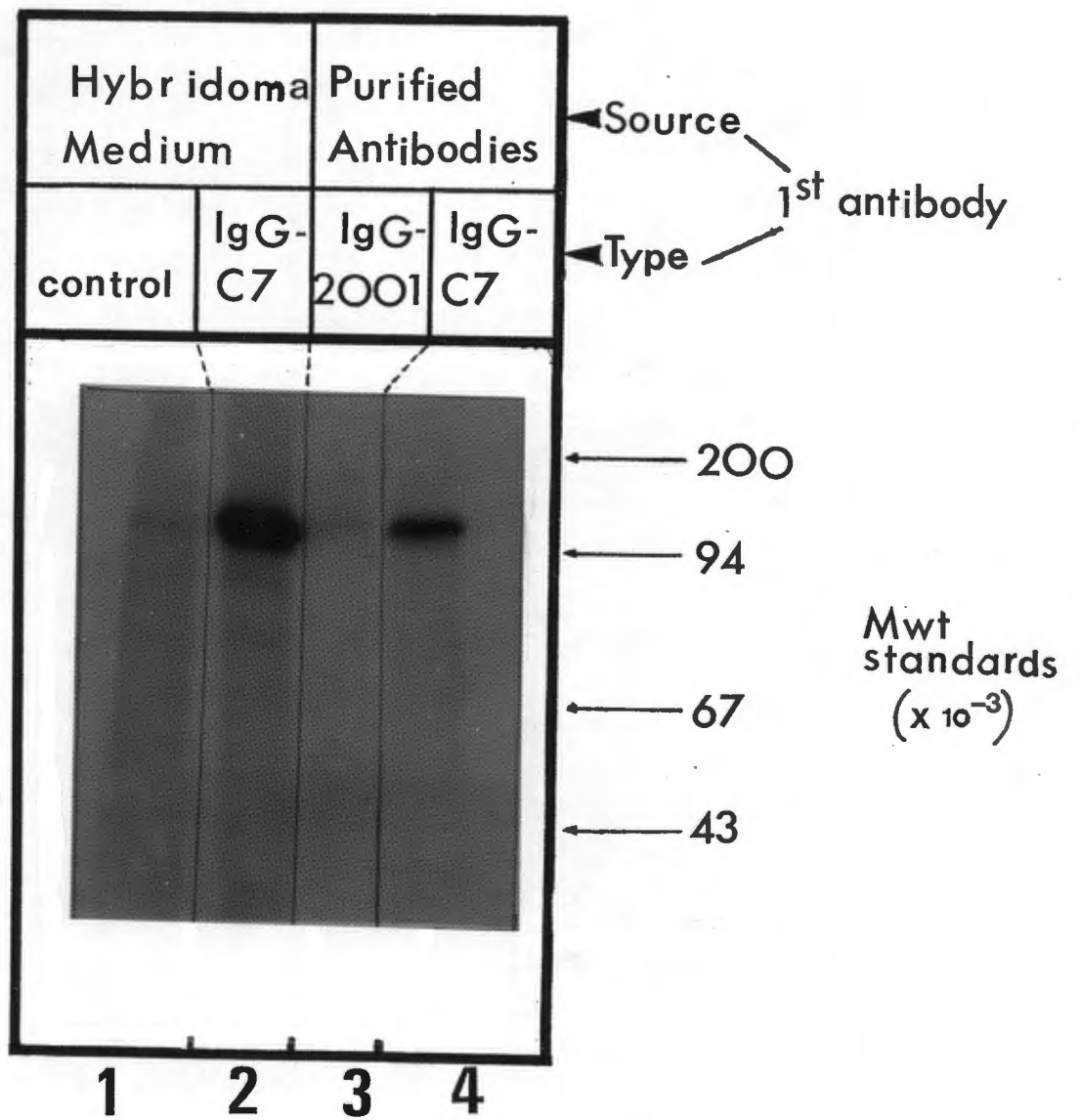
The hybridoma cells were seeded into 35 mm petri dishes at  $0.17 \times 10^6$  cells/ml (2 ml/dish) in standard growth medium. At the indicated times, the cells in each set of dishes were aspirated and counted in a Coulter Counter. A: No fresh medium was added throughout the experiment. B: At each time point from 24 hours onwards, the medium on each of the remaining dishes was replaced with an identical volume of fresh growth medium. Values represent means  $\pm$  S.D. obtained from triplicate dishes.

ethanol, 60  $\mu\text{g/ml}$  penicillin G and 100  $\mu\text{g/ml}$  streptomycin sulphate. General growth characteristics were as described for the IgG-C7 producing hybridoma cell line.

#### Testing the hybridoma cell culture media for antibody production.

The specificity of the antibodies produced by each of the hybridoma cell lines was tested by immunoblotting (22). Partially purified bovine adrenal receptors (209) were subjected to electrophoresis on SDS polyacrylamide gels under non-reducing conditions. Coomassie blue staining of the gels showed many bands (data not given), indicating that the preparation contained many different proteins. The proteins were then electrophoretically transferred from the SDS gel to nitrocellulose paper, and incubated with medium from each of the hybridoma cell lines (Fig. 3.2, lanes 1 & 2), followed by a subsequent incubation with radiolabeled second antibody ( $^{125}\text{I}$ -rabbit anti-mouse IgG). Incubations were also performed using the purified monoclonal antibody IgG-C7, and IgG-2001 - the latter is a mouse monoclonal antibody directed against an irrelevant antigen (*Hemophilus influenzae* type B) - (both antibody types were obtained as gifts from Drs. Brown and Goldstein [Dallas, Texas, U.S.A.]), as first antibodies (Fig 3.2, lanes 4 & 3 respectively). These latter lanes were used as standards against which to compare the hybridoma media results. A single band of mwt 130 kd was observed using either the IgG-C7 hybridoma cell medium or the purified monoclonal antibody (Fig. 3.2, lanes 2 & 4), and was absent from the lanes where incubations had been performed with

FIGURE 3.2



**Fig. 3.2: Immunoblotting of LDL receptor from bovine adrenal cortex to test the hybridoma cell media for antibody production.**

LDL receptors from bovine adrenal cortex were solubilized with octylglucoside and partially purified on DEAE-cellulose (209). 50  $\mu$ g/well was subjected to electrophoresis in the presence of SDS on a 5-20% polyacrylamide gel (non-reducing conditions). The proteins in the gel were transferred electrophoretically to nitrocellulose paper (200 mA, 2 hr, room temp) using a modification of the procedure of Burnette (35). The nitrocellulose paper was cut into strips, blocked (4°C, overnight) in a buffer containing 50 mM TrisHCl, 2 mM  $\text{CaCl}_2$ , 90 mM NaCl, 50 mg/ml BSA, pH 8, and then incubated (room temp, 60 mins) with medium in which the IgG-C7 or control hybridoma cells were grown (lanes 2 & 1) or monoclonal anti-receptor IgG (400 ng/ml) or IgG-2001 (monoclonal antibody directed against an irrelevant antigen) (400 ng/ml) (lanes 4 & 3). Each of the strips was washed with a buffer containing 0.1% Triton X100, 10 mM Tris, 90 mM NaCl, 1% BSA, pH 8, then incubated with  $^{125}\text{I}$ -rabbit anti-mouse IgG ( $8 \times 10^6$  cpm/ml; iodination performed as detailed in section 3.3.2) (room temp, 30 mins). The strips were finally washed, dried and exposed for 20 hours to Cronex 4 X-ray film.

antibodies directed against irrelevant antigens (Fig. 3.2, lanes 1 & 3). This protein (mwt 130 kd under non-reducing conditions) has been purified by others to homogeneity from the bovine adrenal gland, and has been shown to be the LDL receptor (47, 223).

### 3.2.2 Ascites fluid formation.

Since large amounts of antibody were needed for the formation of the immune complexes to be used in the experiments, it was decided to raise ascites fluid, and to purify the monoclonal antibody from this. (Tissue culture supernatant from hybridoma cells contains antibody at approximately 5-10  $\mu\text{g/ml}$ , while the antibody titre in the ascites fluid of immunized mice is usually 100-1000 times higher than this). The procedure outlined below was carried out for both the IgG-C7 hybridoma cell line, and that which produced antibody to an irrelevant antigen.

Female Balb/c mice (6-8 weeks old) were used. At least 10 days before injecting the hybridoma cells, each mouse was injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane). This compound is a tumour promoting agent, and priming the mice in this way increases the frequency of ascites tumour formation (123, 155, 164). The effect of pristane appears to be permanent.

Hybridoma cells were grown and harvested for injection while the cells were growing very actively in the logarithmic phase. The cells were aspirated off the dishes, centrifuged (2 000 rpm, 1 min, room temp), and gently washed once by centrifugation in DMEM only to remove any serum from the medium prior to injection. Final resuspension of the cells was in DMEM only, at  $2 \times 10^6$  to  $5 \times 10^6$  cells per 0.5 ml. Each primed mouse was injected intraperitoneally with 0.5 ml of hybridoma cells. Within 14-21 days, ascitic tumours developed in most of the injected mice. The mice were sacrificed by cervical dislocation, the peritoneal cavities opened, the ascites fluid aspirated and then centrifuged (2 000 rpm, 15 mins, room temp) to remove all tumour cells. The ascites fluid was stored at 4°C in the presence of 0.02% sodium azide.

### **3.3 Purification of IgG-C7.**

#### **3.3.1 General procedure.**

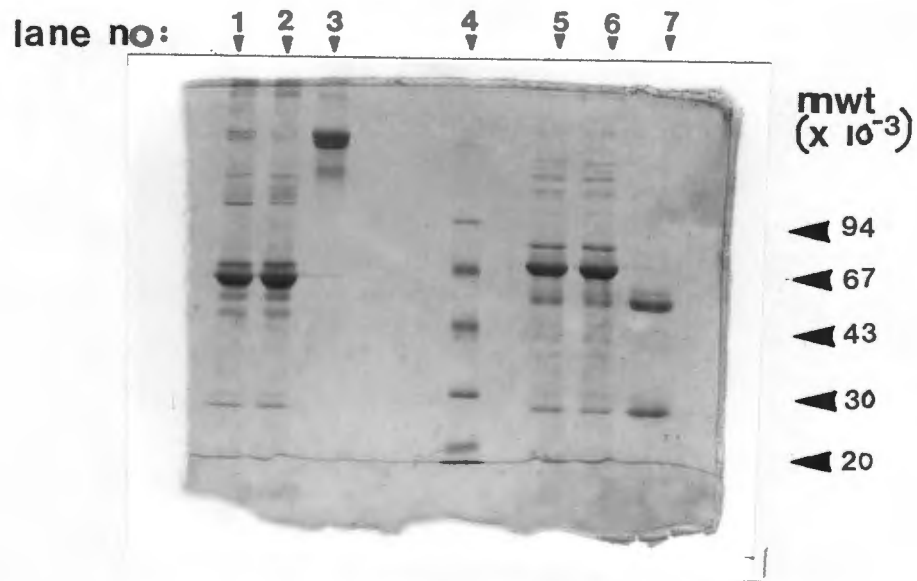
The ascites fluid was applied to columns of Protein A-Sepharose CL-4B that were equilibrated with 0.1M sodium phosphate, pH 7.4 (164). After all the unbound protein had been eluted in a single large peak (pH 7), the pH was reduced to 3 with a solution containing 1 M acetic acid and 0.1 M glycine. At this pH, the IgG protein was eluted, after which its pH was immediately raised to 7.4 by adding the appropriate volume of 2 M Tris. The IgG fraction was dialysed against buffer containing 10 mM sodium phosphate,

50 mM NaCl, pH 7.4 for 24 hr at 4°C, and was then concentrated to 3 mg/ml using an 8MC Amicon Micro-ultrafiltration system. The antibody containing solutions were aliquoted and stored under liquid nitrogen; the protein was never frozen and thawed more than once before use. Yields of purified monoclonal antibody averaged 2-3 mg protein per mouse. The control monoclonal antibody was purified by analogous procedures. Unless otherwise specified, the term "IgG-C7" will be used to refer to the monoclonal antibody to the LDL receptor that was isolated and purified in this laboratory. Similarly, the term "control monoclonal antibody" will be used with reference to the monoclonal antibody directed against the irrelevant antigen (testosterone) which was purified by ourselves.

### 3.3.2 Assessment of the purification process.

#### SDS-PAGE.

In order to judge the efficiency of the affinity-chromatographic step in the monoclonal antibody purification, samples of ascites fluid, the protein peak which eluted at pH 7 and the final peak which eluted at pH 3, were subjected to 5-20% PAGE in the presence of SDS (using both reducing and non-reducing conditions). Fig. 3.3 is representative of a typical purification sequence. The unpurified ascites fluid contained some intact IgG (mwt of 150 kd under non-reducing conditions) and a large amount of impurities, mainly albumin (lane 1). The peak which was eluted from the column at pH 7 (lane 2), contained most of



**Fig. 3.3:** SDS-PAGE of the sequential steps in the purification of IgG-C7, the monoclonal antibody to the LDL receptor.

Samples of the ascites fluid, the peak which eluted from the Protein A-Sepharose CL-4B column at pH 7 (containing the unbound proteins) and the peak which was eluted at pH 3, were subjected to 5-20% SDS-PAGE according to the method of Laemmli (137). Lanes 1, 2 and 3 represent the ascites fluid, the pH 7 peak and the pH 3 peak, respectively, when run under non-reducing conditions, while lanes 5, 6 and 7 show these proteins after electrophoresis under reducing conditions. The positions of the low molecular weight marker proteins are shown in lane 4.

the impurities that were present in the ascites, but very little intact IgG protein was detected. Almost pure IgG eluted from the column at pH 3 (lane 3). When similar samples were run under reducing conditions, both the ascites fluid and the peak which eluted at pH 7 (lanes 5 & 6, respectively) contained relatively small amounts of 25 and 50 kd protein bands. These bands, which represent the reduced form of the intact IgG molecule, were the major components of the pH 3 peak (reduced) (lane 7).

#### Immunoblotting.

The monoclonal antibodies were labeled with  $^{125}\text{I}$  by incubation with Iodogen, a solid-phase iodinating reagent, using a method based on that described by Beisiegel et al (23). Typically, 300  $\mu\text{g}$  protein in 300  $\mu\text{l}$  of buffer (50 mM NaCl, 10 mM sodium phosphate, pH 7.4) and 300  $\mu\text{Ci}$  carrier-free sodium [ $^{125}\text{I}$ ]iodide were added to a glass scintillation vial containing 33  $\mu\text{g}$  of absorbed Iodogen. After incubation at 4°C for 20 mins, Sephadex G-25 column chromatography was used to separate  $^{125}\text{I}$ -labeled antibody from free  $^{125}\text{I}$ . The fractions containing the  $^{125}\text{I}$ -antibody were pooled, and dialysed overnight at 4°C against 5 litres of buffer containing 50 mM NaCl, 10 mM sodium phosphate, pH 7.4. Yields, as measured by protein recovered, averaged 50%, and the specific radioactivity of the labeled antibodies ranged from 1 000 to 1 500 cpm/ng protein. This procedure was also used to iodinate the rabbit anti-mouse IgG which was used as the second antibody in immunoblotting. LDL was radiolabeled with  $^{125}\text{I}$  by the iodine monochloride method as described in section 2.2.3.

To test the ability of the proteins fractionated during the antibody purification procedure to recognise the LDL receptor, the LDL receptor from bovine adrenal cortex was immunoblotted, as described in the legend to Fig. 3.2. As was also reported above (Fig. 3.2), a single band (mwt = 130 kd under non-reducing conditions) was observed when medium from the IgG-C7 producing hybridoma cell line was used as first antibody (Fig. 3.4, lane 1). When the ascites fluid was used in the first incubation, an identical band was detected (lane 2). The monoclonal antibody produced from this ascites fluid, as well as the purified IgG-C7 received as a gift from Brown and Goldstein, also showed the 130 kd protein (lanes 3 & 4). No bands were detected on the autoradiogram when the control monoclonal antibody was used as the first antibody (lane 5). This 130 kd band has been conclusively identified as the LDL receptor by others (47, 223).

#### Binding kinetics of $^{125}\text{I}$ -labeled monoclonal antibodies to human skin fibroblast monolayers.

Concentration curves for the binding of  $^{125}\text{I}$ -LDL and  $^{125}\text{I}$ -IgG-C7 to human skin fibroblast monolayers at 4°C are shown in Figures 3.5A and 3.5B, respectively. For each of the labeled ligands used, the curve was found to have two components: a high-affinity component that approached saturation at an LDL concentration of 10 nM (5 µg/ml) (Fig. 3.5A), or a  $^{125}\text{I}$ -IgG-C7 concentration of 3 nM (0.45 µg/ml) (Fig. 3.5B), and a non-specific component that increased linearly at concentrations of  $^{125}\text{I}$ -LDL up to 50 nM

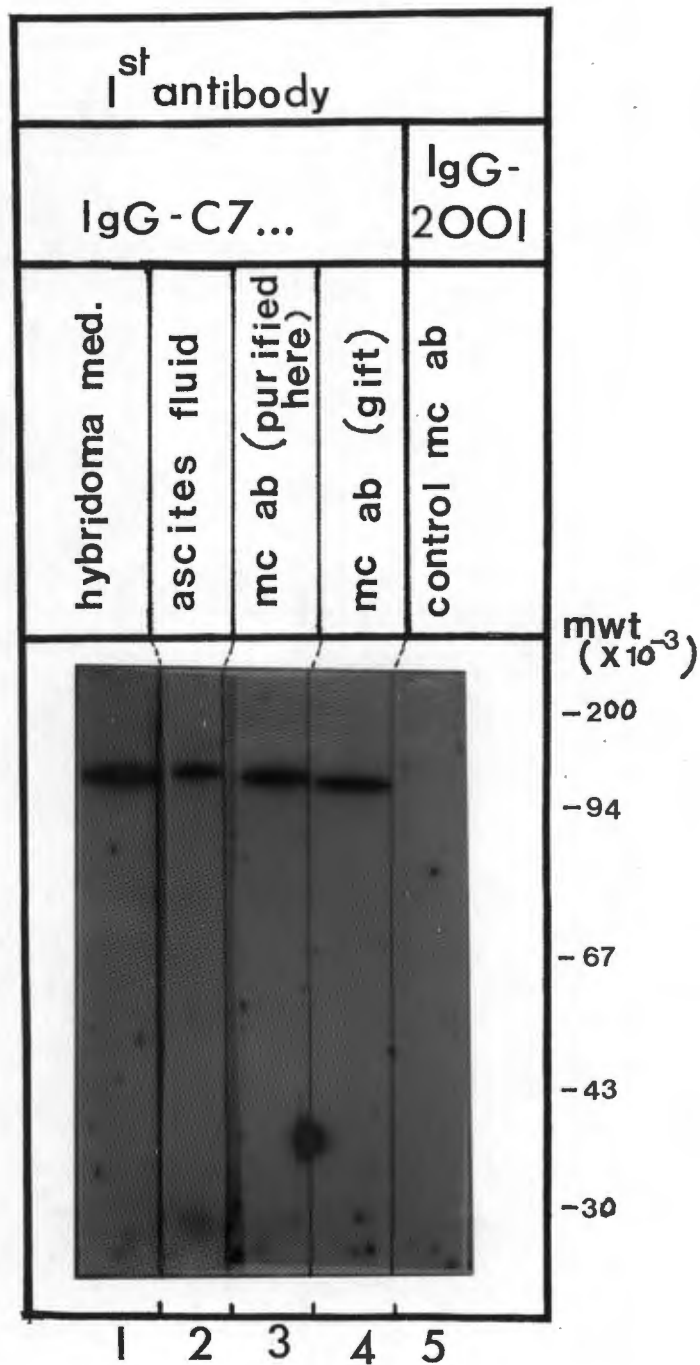


Fig. 3.4: Immunoblotting of the LDL receptor from bovine adrenal cortex, to test the purification procedure.

Immunoblotting of the LDL receptors was performed exactly as described in the legend to Fig. 3.2, except that all monoclonal antibodies were used at 250 ng/ml in the first incubation (lanes 3, 4 and 5). The hybridoma medium and the ascites fluid were diluted 1:1 and 1:75, respectively, with blocking buffer prior to use (lanes 1 & 2).

FIGURE 3.5

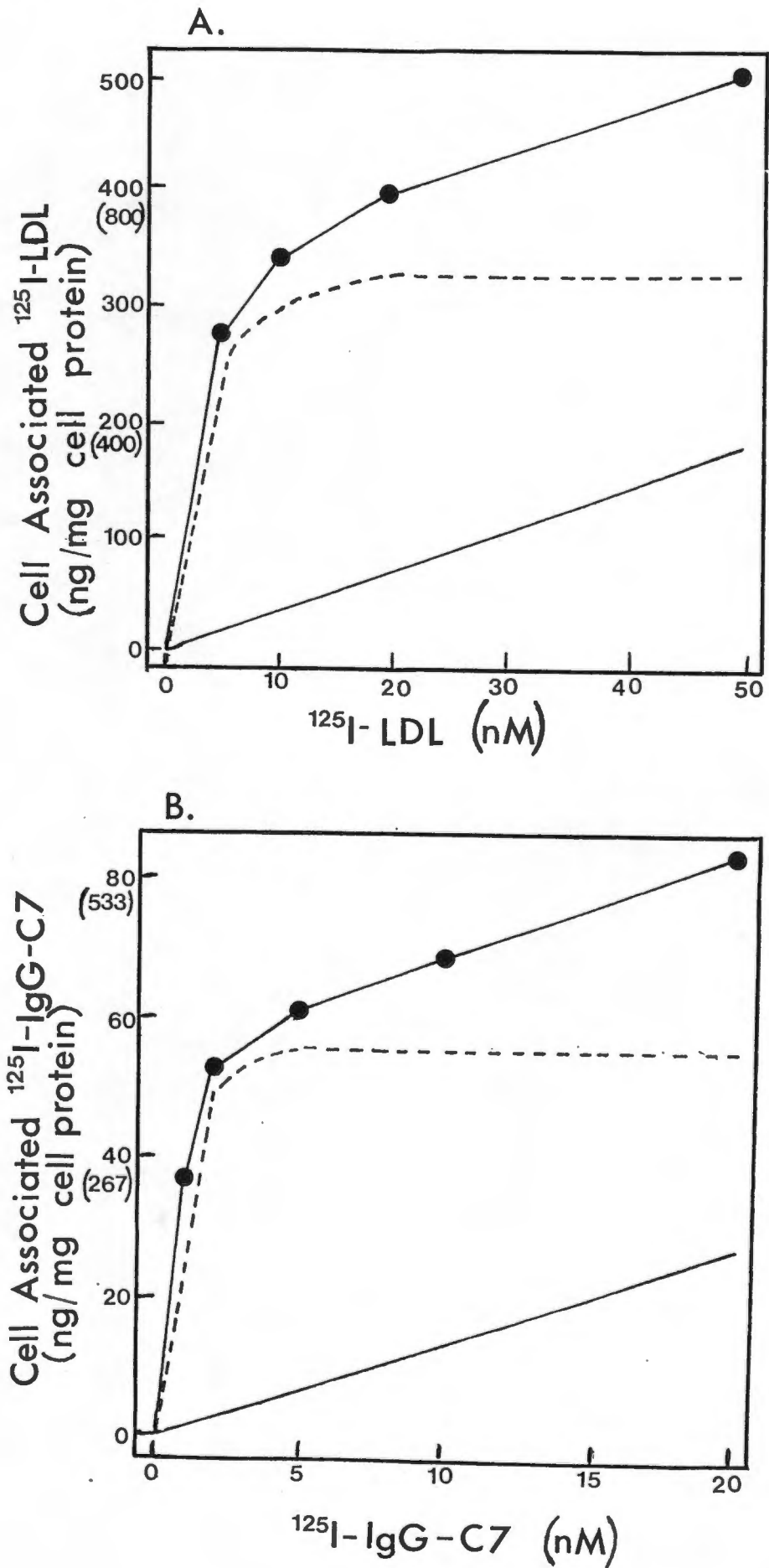


Fig. 3.5: Concentration curves for the binding of (A)  $^{125}\text{I}$ -LDL and (B)  $^{125}\text{I}$ -IgG-C7 to human skin fibroblast monolayers at 4°C.

Normal fibroblast monolayers were grown for 5 days, after which LDL receptors were upregulated in DMEM/LPDS for 48 hr. On day 7 of cell growth, each monolayer was chilled to 4°C, and incubated with 2 ml of ice-cold MEM without bicarbonate, supplemented with 10 mM HEPES and 5 mg protein/ml LPDS, containing the indicated concentration of either  $^{125}\text{I}$ -LDL (570 cpm/ng protein) or  $^{125}\text{I}$ -IgG-C7 (1 300 cpm/ng protein) for 2 hr at 4°C. The cells were then washed and solubilized in NaOH as described in section 2.2.7. Total binding was obtained by incubating in the presence of the appropriate radiolabeled ligand only. In each case, the low-affinity component was derived by extrapolation from the slope of the terminal linear portion of the relevant concentration curve. The dashed lines show the high-affinity binding, and were obtained using the slope-peel technique as described by Anderson *et al* (3). Experimental points are the means of duplicate dishes; these values did not differ from the means by more than 5%. The data are taken from one of three representative experiments. Molecular weights of 500 000 and 150 000 were used for LDL protein and IgG-C7 protein, respectively, in order to calculate stoichiometry. The figures in parentheses along the y-axis in each plot are the cell-associated values calculated in fmol/mg cell protein.

(25  $\mu\text{g/ml}$ ) (Fig. 3.5A) and  $^{125}\text{I}$ -IgG-C7 up to 20 nM (3  $\mu\text{g/ml}$ ) (Fig. 3.5B). The dashed lines in the figures represent the high-affinity binding, obtained using the slope-peel technique (3) as detailed in the legend to the figure. The  $K_D$  values for  $^{125}\text{I}$ -LDL and  $^{125}\text{I}$ -IgG-C7 binding to the high-affinity receptors were 3.6 nM (1.8  $\mu\text{g/ml}$ ) (Fig. 3.5A), and 0.5 nM (0.075  $\mu\text{g/ml}$ ) (Fig. 3.5B), respectively. These values were calculated as described in section 2.2.9. Approximately equimolar amounts of  $^{125}\text{I}$ -LDL and  $^{125}\text{I}$ -IgG-C7 were bound to the cells at saturation (about 400 fmol/mg cell protein), indicating one high-affinity monoclonal antibody binding site for each high-affinity LDL binding site. These data correspond exactly with those obtained by others, also using the monoclonal antibody to the LDL receptor (22, 23, 243). When the control monoclonal antibody was iodinated and incubated with human skin fibroblast monolayers in an identical manner, virtually no high-affinity binding was detected (data not shown).

#### Inhibition of $^{125}\text{I}$ -LDL binding to cells by prior incubation of cells in the presence of IgG-C7.

The monoclonal antibody, IgG-C7, was able to inhibit to some extent, the binding of  $^{125}\text{I}$ -LDL to the LDL receptor of human skin fibroblasts. When intact cell monolayers were subjected to prior incubation for various times with the anti-LDL receptor antibody (0 to 120 mins), subsequent  $^{125}\text{I}$ -LDL binding (1 hr at 4°C) was found to be decreased maximally (50%) after pre-incubation times of 90 mins or longer (data not shown). Similar pre-incubations using the control monoclonal antibody directed against an irrelevant

antigen were shown to have no effect on the amount of  $^{125}\text{I}$ -LDL bound to the cells. Inhibition of  $^{125}\text{I}$ -LDL binding to the cells at  $4^\circ\text{C}$  using a range of unlabeled IgG-C7 concentrations showed a 40% decrease in binding at 2 nM IgG-C7 (Fig. 3.6). Complete inhibition of  $^{125}\text{I}$ -LDL binding was not achieved (a 50% decrease in binding was observed at 50 nM IgG-C7, the highest concentration used). When  $^{125}\text{I}$ -IgG-C7 was bound to the cells in the presence of LDL (30  $\mu\text{g}/\text{ml}$ ), after a prior incubation for 1 hr at  $4^\circ\text{C}$  with LDL, no inhibition was detected (data not shown). These effects have also been documented by others (21, 23). The exact molecular explanation of the binding behaviour of these two ligands awaits clarification.

### 3.3.3 Other antibodies used in this study.

The polyclonal antibody to the LDL receptor was a generous gift from Drs. Brown and Goldstein (Departments of Molecular Genetics and Internal Medicine, University of Dallas Health Science Centre, Dallas, Texas, U.S.A.). It was raised in rabbits against a purified LDL receptor preparation from bovine adrenal cortex, as detailed in reference 21, and has been shown to cross react with the partially purified receptor preparation that was used to immunize the rabbits. This antibody is directed against the entire LDL receptor protein, in contrast to the monoclonal antibody, IgG-C7, which recognizes only the N-terminal LDL receptor binding domain.

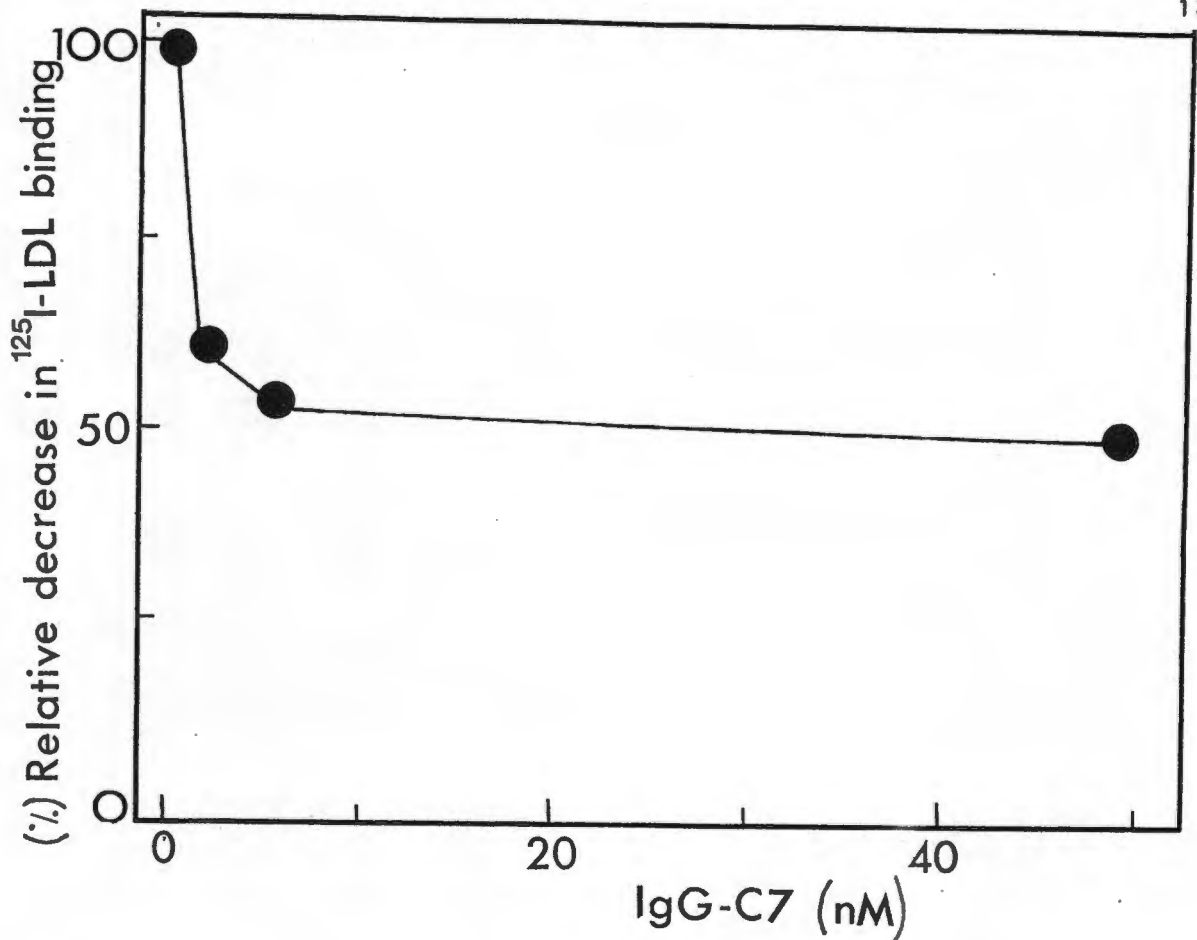


Fig. 3.6: Inhibition of <sup>125</sup>I-LDL binding to human skin fibroblasts at 4°C by IgG-C7.

Normal fibroblasts were grown and upregulated as described in the legend to Fig. 3.5. On day 7 of cell growth, each monolayer was chilled to 4°C, washed and incubated for 2 hr at 4°C in MEM without bicarbonate supplemented with 10 mM HEPES and 5 mg protein/ml LPDS containing the indicated concentration of IgG-C7. After this time, <sup>125</sup>I-LDL (7 µg/ml) was added to each dish, and binding was effected for 1 hr at 4°C. The cells were washed and harvested in NaOH as described in section 2.2.7. Experimental points are the means of duplicate dishes which did not differ from each other by more than 5%. Data plotted represent the <sup>125</sup>I-LDL bound in the presence of IgG-C7, expressed as a percentage of the labeled ligand which was bound in the absence of IgG-C7.

B3/25, a monoclonal antibody to the transferrin receptor, was a generous gift from Dr. I.S. Trowbridge (Dept. of Cancer Biology, The Salk Institute for Biological Studies, San Diego, California, U.S.A.). This antibody was obtained from the fusion of Balb/c mice spleen cells, immunized against the human hematopoietic cell line K562, with a myeloma cell line (174).

### 3.4 Immune complex formation.

#### 3.4.1 Method.

All monoclonal antibodies (IgG-C7, B3/25 and the control monoclonal) were aliquoted and stored under liquid nitrogen in a 50 mM sodium phosphate buffer, pH 7.4, at 3 mg/ml. The polyclonal antibody was stored at  $-70^{\circ}\text{C}$ . Preformed immune complexes containing IgG-C7, B3/25 or the control monoclonal antibody and a second antibody (goat anti-mouse IgG, in each case - specific for heavy- and light-chains) were prepared using the method described by Tolleshaug et al (243), with a few further modifications. The following reagents were used:

IgG-C7 (or B3/25 or control monoclonal): 150  $\mu\text{g}$  in 50  $\mu\text{l}$  sodium phosphate buffer.

Goat anti-mouse IgG: 1.5 mg in 150  $\mu\text{l}$  of 20 mM PBS, pH 7.4, with 0.05% sodium azide.

Immune complex buffer: 150  $\mu\text{l}$ , containing 50 mM TrisHCl, pH 8; 200 mM NaCl, 1 mM EDTA and 0.5% (v/v) nonidet P-40.

Reaction mixtures were incubated in microfuge tubes at room temperature for 30 mins, then at 4°C for 24 hours. After incubation, the tubes were spun briefly (2 000xg, 4°C, 30 sec), and the supernatant fluid above the precipitate was removed. Precipitates were resuspended in 1 ml of immune complex buffer, and spun briefly as before. The immunoprecipitates were then dispersed in 500 µl of immune complex buffer by repeated passage through a pipette tip, stored at 4°C, and used within 2 weeks. Preformed immune complexes using either the polyclonal antibody or the nonimmune IgG were prepared similarly, except that goat anti-rabbit IgG was used as the second antibody for both, because these antibodies had been raised in rabbits.

Protein concentrations of the resuspended immune complexes ranged from 1.5 to 3 mg/ml. Yields were typically 70-80% with respect to total protein used.

#### 3.4.2 Optimization of conditions for immune complex formation.

##### Time course of precipitation.

Immune complexes were made as described above, using <sup>125</sup>I-IgG-C7 (specific activity = 1 300 cpm/ng protein), goat anti-mouse IgG, and immune complex buffer. Control tubes contained the identical components, except that the goat anti-mouse IgG was omitted. After incubation for 30 mins at room temperature, the mixtures were kept at 4°C for various times (1 day, 1 1/2 days or 4 days). At each of these times,

the tubes were centrifuged briefly, and 10  $\mu$ l of supernatant was counted in a gamma counter. All points were done in duplicate, and values differed from each other by less than 5%. Results obtained showed an 87% precipitation of  $^{125}\text{I}$ -IgG-C7 at day 1, which stayed constant at the 1 $\frac{1}{2}$  and 4 day times. In all subsequent experiments, immune complex formation (4°C) was for 24-48 hours.

#### Optimum antibody ratios in the immune complex.

Immune complexes were made essentially as described in section 3.4.1, with each tube containing an identical amount of  $^{125}\text{I}$ -IgG-C7 (specific activity = 1 300 cpm/ng protein), but varying amounts of goat anti-mouse IgG protein, in order to obtain a range of ratios of first to second antibodies. The ratios used varied from 1:80 ( $^{125}\text{I}$ -IgG-C7 : goat anti-mouse IgG) to 1:1. In order to keep the total volume of the immune complex mixture constant, the volume of immune complex buffer added in each tube was adjusted accordingly. Control tubes contained the radiolabeled IgG-C7 and the immune complex buffer only. At various times (24 hr, 48 hr or 66 hr), the percentage of the  $^{125}\text{I}$ -IgG-C7 which had precipitated relative to the control was calculated as described above. The results, which are shown in Table 3.1, indicate that when the goat anti-mouse IgG was present in an excess of 10-fold (or greater), more than 80% of the  $^{125}\text{I}$ -IgG-C7 precipitated in the immune complex, irrespective of the length of the 4°C incubation. All subsequent immune complexes were made using a 1:10 ratio of IgG-C7 : goat anti-mouse IgG.

**Table 3.1: Optimization of antibody ratios in the immune complex.**

Immune complexes, containing varying amounts of goat anti-mouse IgG and a constant amount of  $^{125}\text{I}$ -IgG-C7, were made and analysed at the indicated times, as described in section 3.4.2. The results were calculated from single measurements at each time point.

Ratio of $^{125}\text{I}$ -IgG-C7:G anti-M*	% Precipitation of $^{125}\text{I}$ -IgG-C7		
	Time (hours)		
	24	48	66
1 : 80	80	82	83
1 : 40	85	86	87
1 : 20	85	89	90
1 : 10	81	91	89
1 : 5	nd**	nd	nd
1 : 2.5	nd	nd	nd
1 : 1	nd	nd	nd

\* =  $^{125}\text{I}$ -IgG-C7:goat anti-mouse complex

\*\* = not detectable

### 3.5 Cells - growth, labeling and solubilization.

#### 3.5.1 Cell growth and LDL receptor upregulation.

Unless otherwise specified, human skin fibroblasts were set up in 60 mm petri dishes according to the standard format described in section 2.2.1. The cells were routinely maintained in growth medium for 4 days only before initiating upregulation of the LDL receptors by incubating the monolayers in DMEM/LPDS, as described in section 2.2.5. Upregulation was effected for 16 hr prior to commencing the pulse-label, unless otherwise specified.

#### 3.5.2 Biosynthetic labeling of cell proteins.

After the LDL receptors on the monolayers had been upregulated, the cells were washed once with 2 ml/dish of PBS, and then incubated with 1.3 ml/dish of methionine-free MEM (EMEM) containing 5 mg/ml LPDS and the indicated amounts of [ $^{35}$ S]-methionine (the pulse) [Note: The source of [ $^{35}$ S]-methionine used in the relevant experiments was Tran $^{35}$ S-label, which was the hydrolysate of E. coli grown in the presence of  $\text{H}_2^{35}\text{SO}_4$ , and contained [ $^{35}$ S]-methionine, 70%; [ $^{35}$ S]-cysteine, 20%; [ $^{35}$ S]-methionine sulphoxide, 7%; [ $^{35}$ S]-cysteic acid, 2% and other  $^{35}\text{S}$ -compounds, 1%. In all cases, the amounts of  $^{35}\text{S}$ -label added to the media have been expressed in terms of the actual [ $^{35}$ S]-methionine content. For convenience, the label will be referred to hereafter as

[<sup>35</sup>S]-methionine]. Pulse-labeling was performed for 8 hr unless otherwise specified. The pulse medium was then removed, after which the cell monolayers were washed once with PBS, then chased with 2 ml/dish of DMEM/LPDS (containing 200  $\mu$ M methionine) for various times at 37°C, unless otherwise indicated. In those cases where the cells were not chased subsequent to labeling, the dishes - still containing the pulse medium - were processed for solubilization immediately.

In order to assess the effect of the methionine-free medium on cell viability, the monolayers were grown and upregulated as described in section 3.5.1. The cells were then incubated either in fresh DMEM/LPDS, or in methionine-free medium (EMEM) supplemented with LPDS and 10  $\mu$ M cold methionine (an amount well below the lowest amount of [<sup>35</sup>S]-methionine used in any of the pulse procedures). At the indicated times,  $2.2 \times 10^7$  dpm of [<sup>3</sup>H]-phenylalanine was added to each dish, and the incubations were continued for a further 2 hours at 37°C. Protein synthesis rates at each of these times was then determined after TCA precipitation. The results, which are depicted in Fig. 3.7, show that there was no significant difference in protein synthesis rates and hence, by extrapolation, cell viability, over the 50 hour period which was tested. The rates in both cases decreased as a function of time, probably due to general "step-down" conditions imposed by the lack of lipoproteins in the medium.

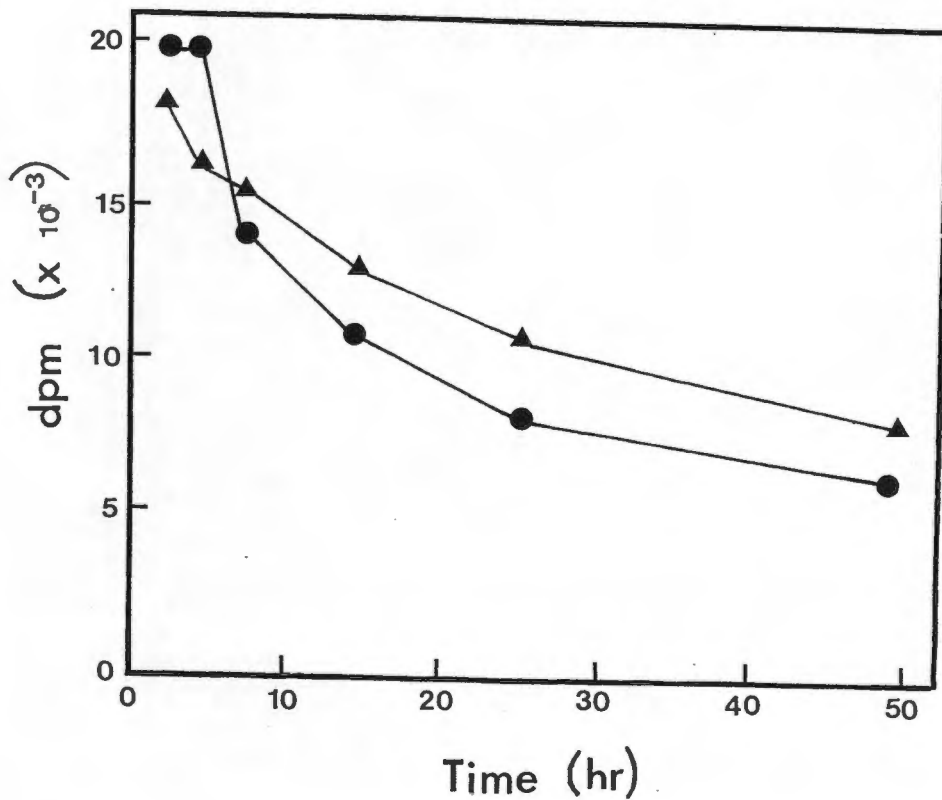


Fig. 3.7 The effects of DMEM/LPDS (●), or EMEM/LPDS supplemented with methionine (▲), on protein synthesis in fibroblasts.

Cells were grown, upregulated and incubated in DMEM/LPDS (●) or EMEM/LPDS supplemented with 10  $\mu$ M cold methionine (▲) as described in section 3.5.2. Protein synthesis rates were measured at the indicated times after TCA precipitation. The results are means obtained from duplicate dishes at each time point. Values never differed by more than 5% from the means.

### 3.5.3 Cell solubilization.

After the indicated chase periods, the cell monolayers were chilled to 4°C for 10 mins, and washed twice with 2 ml of 4°C buffer containing 10 mM Hepes, pH 7.4, 150 mM NaCl and 2 mM CaCl<sub>2</sub>. The cells were then lysed by addition of 200 µl of ice-cold Buffer B containing 10 mM Hepes, pH 7.4, 200 mM NaCl, 2 mM CaCl<sub>2</sub>, 2.5 mM MgCl<sub>2</sub>, 1 mM PMSF in 0.25% (v/v) dimethylsulphoxide, 0.1 mM leupeptin and 1% (v/v) Triton X-100. The cells were scraped from each dish with a teflon policeman, transferred to 1.5 ml microfuge tubes, and the dishes were washed with a second 200 µl volume of Buffer B; this was pooled with the original harvest. Centrifugation was performed at 4°C for 10 mins at 12 000xg in a microfuge. The pellets were discarded, and the cell extracts (320 µl out of the total volume obtained from one dish of cells) were either processed immediately, or were frozen under liquid nitrogen before immunoprecipitation. This latter procedure was generally followed since it enabled an entire pulse-chase experiment to be immunoprecipitated and processed simultaneously after solubilization at the initial staggered chase times. The effect of freezing the detergent-solubilized cell extracts for various lengths of time before immunoprecipitation, was examined. Irrespective of whether the extracts were frozen for 4, 8, 24 or 48 hours before thawing and subsequent immunoprecipitation, or whether they were frozen and thawed immediately, similar amounts of LDL receptor protein were immunoprecipitated when compared to solubilized cells which had never been frozen. Since the freeze-thaw procedure

produced no significant loss of LDL receptor activity, it was used routinely to facilitate subsequent experimentation.

### 3.6 Immunoprecipitation

#### 3.6.1 Method

The immunoprecipitation method used, was based on the procedure originally described by Terhorst et al (240), as modified by Tolleshoug et al (244), with a few additional alterations. 320  $\mu$ l of  $^{35}$ S-labeled cell extract was mixed with a 40  $\mu$ l or an 80  $\mu$ l aliquot of one of the preformed immune complexes (containing IgG-C7 or control antibody or the polyclonal antibody). The mixture was incubated at 4°C for 1 hour on a rocking platform, and then centrifuged in a Microfuge (2 000 rpm, 4°C, 5 mins). The supernatant above each pellet was either discarded, or was carefully aspirated off and mixed with a second 40  $\mu$ l aliquot of preformed immune complex, then precipitated and processed as per the first immunoprecipitation. The latter procedure was mainly used for the immunoprecipitation of the transferrin receptor (ie. the supernatant from the first precipitation was mixed with preformed immune complex containing B3/25, in order to precipitate the transferrin receptor). This procedure was also used to check the effectiveness of the first precipitation (ie. a second precipitation was performed using IgG-C7 containing immune complex).

Each pellet formed during the precipitation step, was dissolved in 200  $\mu$ l of Buffer B, and then layered on top of a four-step sucrose gradient, prepared as described by Tolleshaug et al (243). All sucrose gradients contained 10 mM TrisHCl, pH 8 and 1 mM PMSF. Each layer (750  $\mu$ l) was composed of the following:

Layer 1 (top): 10% sucrose, 0.5% CHAPS  
Layer 2: 20% sucrose, 0.5 M NaCl, 0.2% Nonidet P-40  
Layer 3: 30% sucrose, 0.1% SDS, 0.2% Nonidet P-40  
Layer 4 (bottom) 40% sucrose

The gradients were centrifuged at 2 000 rpm for 30 mins at 4°C in a Beckman TJ-6 centrifuge. Each supernatant was then carefully poured off, and the remaining pellet resuspended in 200  $\mu$ l of buffer containing 50 mM TrisCl, pH 8 and 2 mM CaCl<sub>2</sub>, transferred to a microfuge tube and spun at 12 000 rpm for 20 seconds. This final precipitate was dissolved in 40  $\mu$ l of a solution containing 4 M urea, 100 mM dithiothreitol, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.4% (w/v) SDS and 75 mM Tris, heated to 90°C for 3 mins in a water bath and electrophoresed as described in section 3.7.1.

In one experiment, aliquots of the solubilized cells and aliquots of the final immunoprecipitates were precipitated with TCA. In this way, it was calculated that the labeled LDL receptor protein and the labeled transferrin receptor protein represented approximately 0.035% and 0.06%, respectively, of total labeled cell proteins pulsed for 8 hours.

### 3.6.2 Optimization of the precipitation conditions.

#### Preclearing

There existed the distinct possibility that a first incubation of the cell extracts with an immune complex containing the control monoclonal antibody (directed against an irrelevant antigen), would precipitate, and hence remove, any non-specific radiolabeled proteins - an effective "preclear". Thus subsequent precipitations of the LDL and transferrin receptors would (theoretically) contain almost entirely the desired protein. This procedure was investigated, but was not adopted because it made no difference to the quality of the results obtained when the preclear procedure was omitted (data not shown).

#### The order of sequential immunoprecipitations.

We tested whether the precipitation using the immune complex containing IgG-C7 would be optimized if this immune complex was used either before, simultaneously with, or after that containing the monoclonal antibody to the transferrin receptor. To this end, radiolabeled, detergent-solubilized cell extracts were subjected to successive immunoprecipitations with immune complexes containing monoclonal antibodies, as follows: (i) first IgG-C7, followed by B3/25; (ii) first B3/25, followed by IgG-C7 or (iii) a combination of IgG-C7 + B3/25 in a single precipitation. The latter approach was feasible since the two receptors differed in their apparent molecular weight on SDS-PAGE (LDL receptor = 160 kd; transferrin receptor = 90 kd). The

results showed clearly that in all the above combinations, similar amounts of receptor proteins were immunoprecipitated (data not given). In most experiments reported in this thesis, approach (i) was used, but occasionally approach (iii) was adopted (see legends to figures).

#### **Time required for immunoprecipitation.**

When the length of the immunoprecipitation incubation was varied from 1 hour to 16 hours, no detectable difference was noted in the amount of receptor precipitated (data not shown). However, at the longer time points, the amount of labeled, non-specific proteins which co-precipitated was greatly increased - very noticeable among these were actin (43 kd) and myosin (200 kd). To obviate this problem, precipitations were always done for less than 3 hours - usually for 1 hour at 4°C.

### **3.7 Visualization of the labeled immunoprecipitates.**

#### **3.7.1 SDS-PAGE.**

Electrophoresis was carried out on SDS-polyacrylamide slab gels, according to the method of Laemmli (137), using a linear 5-20% acrylamide gradient. Samples were reduced by incubating (at 90°C, for 3 mins) with 2-mercaptoethanol. Electrophoresis was performed at 30 mA per slab gel, at room temperature, for 3 hours. Gels were calibrated with the following standards: myosin (200 kd), phosphorylase b (94 kd), bovine serum albumin (67 kd), ovalbumin (43 kd),

carbonic anhydrase (30 kd), trypsin inhibitor (20 kd) and alpha-lactalbumin (14 kd). The gels were routinely stained and destained prior to drying, in order to visualize the positions of the molecular weight marker standards, and to check the resolution of protein bands.

### 3.7.2 Fluorography

Destained gels were enhanced with the water soluble fluor, sodium salicylate, dried and subjected to fluorography using the following procedure. The gels were washed in water for 30 mins to prevent the subsequent precipitation of sodium salicylate. They were then soaked for 30 minutes in 1 M sodium salicylate at room temperature according to the method described by Chamberlain (39). At this concentration of fluor, and after soaking for this length of time in the fluor, film darkening was optimal. The gels were then dried for 3 hours at 60°C on a Dual Temperature Slab Gel Dryer, and exposed to a preflashed X-ray film (Kodak XAR-5 film) (139) at -70°C for times ranging from 16 hours to 48 hours.

The rationale behind the use of this method is as follows. The fluor converts the energy of the beta-particles to visible light, which then forms an image on the X-ray film. In this way, absorption of beta-particles within the sample is overcome by the increased penetration of light. Preflashing of the film produces a fluorographic image which is proportional to the distribution of the radioactivity in the sample. At the temperatures used during exposure of the

film ( $-70^{\circ}\text{C}$ ), the sensitivity to low light intensities is increased due to the lengthening of the half-life of single silver atoms, which facilitates the initial stage of latent image formation. The overall image improvement using this method of fluorography over direct autoradiography is about 15-fold (138).

Quantitation of the fluorograms was performed spectrophotometrically as described by Suissa (231). The parts of the film containing the darkened bands were excised as well as pieces of corresponding size from areas which had not been exposed to radioactivity (the blanks). These excised pieces were submerged in 1 ml aliquots of 1 N NaOH (millipore filtered before use) and incubated at room temperature for 2 hours or longer, until all the silver grains had been eluted from the film slices. The solutions were vortexed mixed, and the pieces of clear film removed from each tube. 30% glycerol was added to each, and the tubes were again vortex mixed before reading the absorbances on a spectrophotometer at 500 nm. In all cases, care was taken to expose the fluorograms such that the darkened bands were below the plateau density of film darkening, and hence well within the linear response range of the film. To obtain maximum clarity for photographic reproduction only, duplicate fluorograms were overexposed in some cases.

### 3.7.3 Quantitativeness of the immunoprecipitation and fluorographic procedures.

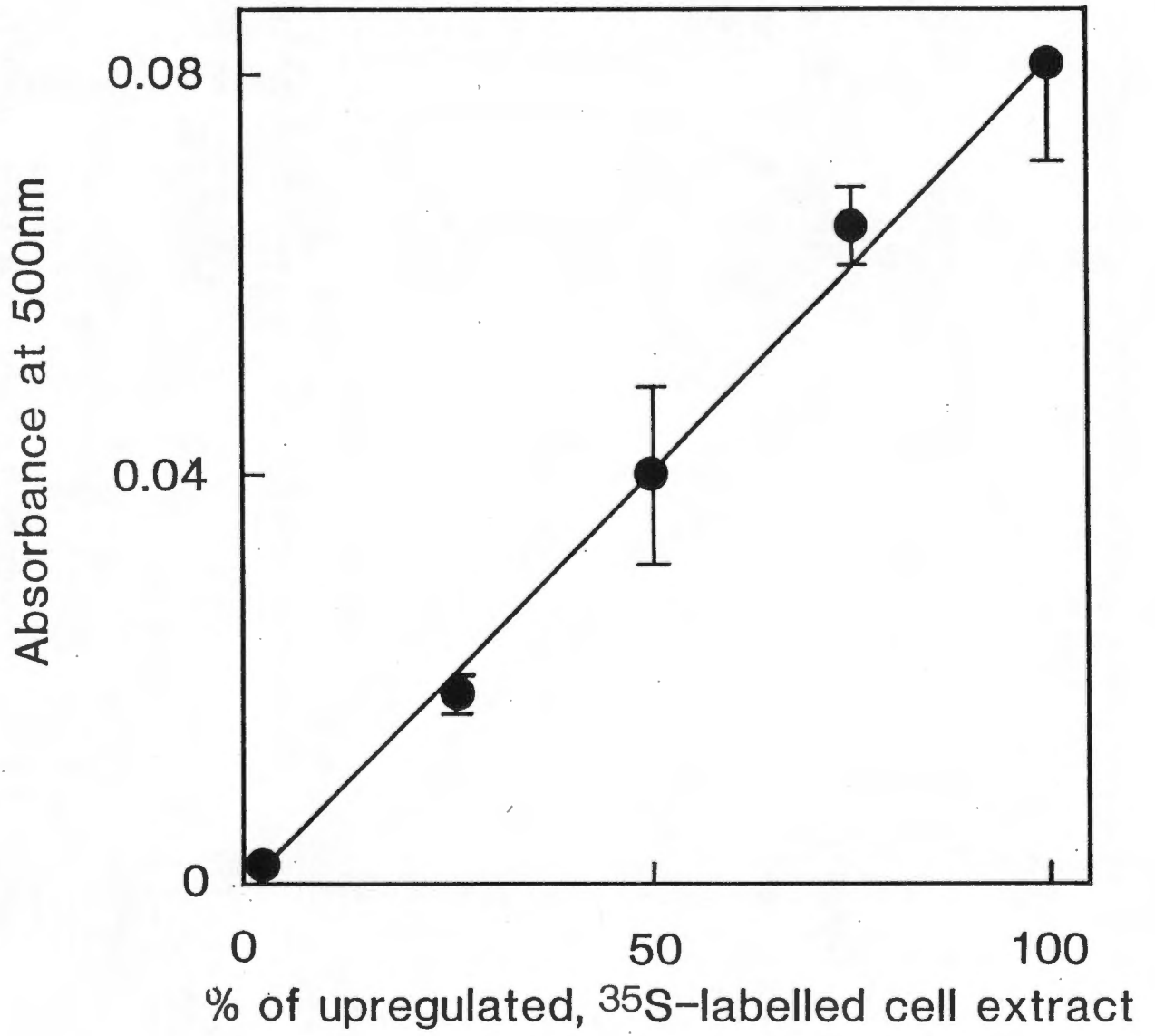
In theory, the proportion of an antigen which reacts with a constant amount of an antibody does not vary with respect to antigen concentration provided that the affinity constant remains the same. This was borne out by a direct test of the relationship between the LDL receptor content of a set of samples, and the amount of radioactivity detected on fluorograms using the standard immunoprecipitation procedure with a constant amount of monoclonal antibody complex (Fig. 3.8). The NaOH extraction method of measuring the density of bands on fluorograms was also shown to be quantitative, provided that care was taken to expose the fluorograms in such a manner that the darkened bands were below the plateau density of film darkening, and hence well within the linear response range of the film. The absorbance readings within an individual experiment were generally in the range of 0.03 - 0.2 units, after subtraction of the film blank value.

### 3.8 Verification of the identity of the immunoprecipitated protein.

#### 3.8.1 Use of an immune complex containing monoclonal antibodies directed against an irrelevant antigen.

It is well documented that the mature LDL receptor has an apparent molecular weight of 160 kd when subjected to SDS-PAGE under reducing conditions (243); that of the

FIGURE 3.8



**Fig. 3.8: Quantitation of immunoprecipitation and fluorogram extraction procedures.**

Normal fibroblast monolayers were incubated either in DMEM/LPDS (to upregulate the LDL receptors), or in DMEM/LPDS + 1  $\mu\text{g/ml}$  of 25-hydroxycholesterol (to downregulate the LDL receptors) for 15 hours. The cells were then pulse-labeled for 8 hours in EMEM/LPDS containing 50  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine in the absence or presence of 1  $\mu\text{g/ml}$  of 25-hydroxycholesterol (for the up- and downregulated monolayers, respectively). Monolayers were detergent solubilized, and 320  $\mu\text{l}$  of the supernatant from each microfuge tube was pooled appropriately to yield a stock of solubilized cell extracts, in which the LDL receptors had been either up- or downregulated. These stocks were mixed in varying proportions to give an accurately known series of diluted radiolabeled LDL receptors. Immunoprecipitation was performed with 40  $\mu\text{l}$  of IgG-C7 containing immune complex per 320  $\mu\text{l}$  of mixture. The labeled immunoprecipitates were then subjected to SDS-PAGE and fluorography. Quantitation of the bands corresponding to the LDL receptors on the fluorograms was done by NaOH extraction. Data shown represent the mean  $\pm$  S.D. of the absorbances obtained from triplicate precipitations. The results are typical of those obtained in 3 such experiments, performed at various times during the course of this study to confirm the quantitiveness of the assay. The linearity of the response was maintained up to about 0.3 absorbance units (using fluorograms which had been exposed for longer times - data not shown).

transferrin receptor is about 90 kd (173). We demonstrated that the immunoprecipitation of the 90 kd and 160 kd proteins did not merely represent non-specific precipitation, by using an immune complex containing the control monoclonal antibody (directed against an irrelevant antigen). Standard procedures were used for the immunoprecipitations and fluorography (Fig. 3.9, lanes 3 & 4). When these results were compared to those obtained using immune complex containing IgG-C7 and B3/25 (Fig. 3.9, lanes 1 & 2), it was evident that both the 160 kd LDL receptor bands, and the 90 kd transferrin receptor bands were specifically immunoprecipitated by the appropriate monoclonal antibodies.

To show that the specifically precipitated 160 kd band actually represented the LDL receptor, the investigations outlined in sections 3.8.2, 3.8.3 and 3.8.4 were performed.

### **3.8.2 Synthesis and processing of the LDL receptor.**

The precursor form of the 160 kd protein was labeled by incubating the fibroblast monolayers for 30 minutes in [<sup>35</sup>S]-methionine containing medium. This short pulse-incubation was followed by chase periods ranging from 0 to 120 minutes, after which the receptors were immunoprecipitated and subjected to SDS-PAGE. The fluorogram was extracted, and the percentage protein in the 120 kd and 160 kd forms at each time was determined (Fig. 3.10). When the cells were pulse-labeled with [<sup>35</sup>S]-methionine for

**FIGURE 3.9**

GM 3348 A			T.T.	Cell line
upregulated		down	up	LDL receptor status
C7 + B3/25	control ab	C7 + B3/25		immune complex
1,2	3,4	5,6	7,8	lane no
				- LDL-R - Tf-R

**Fig. 3.9: Verification of the identity of the 160 kd immunoprecipitated protein.**

Normal human skin fibroblast monolayers (GM 3348A) (lanes 1-6) and those from patient T.T. (see section 3.8.4) (lanes 7 & 8) were cultured as described in section 3.5.1. On day 4 of cell growth, the LDL receptors on the monolayers were either upregulated (by incubation in DMEM/LPDS) or downregulated (by incubation in DMEM/LPDS + 1  $\mu$ g/ml 25-hydroxycholesterol) for 16 hours. The monolayers were then pulse-labeled for 8 hours with EMEM/LPDS containing 40  $\mu$ Ci/ml [ $^{35}$ S]-methionine, in the absence or presence of 25-hydroxycholesterol. After detergent solubilization, the cell extracts were incubated with preformed immune complexes containing IgG-C7 + B3/25 (lanes 1, 2 & 5-8) or the control monoclonal antibody (lanes 3 & 4). The radiolabeled immunoprecipitates were subjected to SDS-PAGE and fluorography. Duplicate precipitations were performed for each of the variables tested.

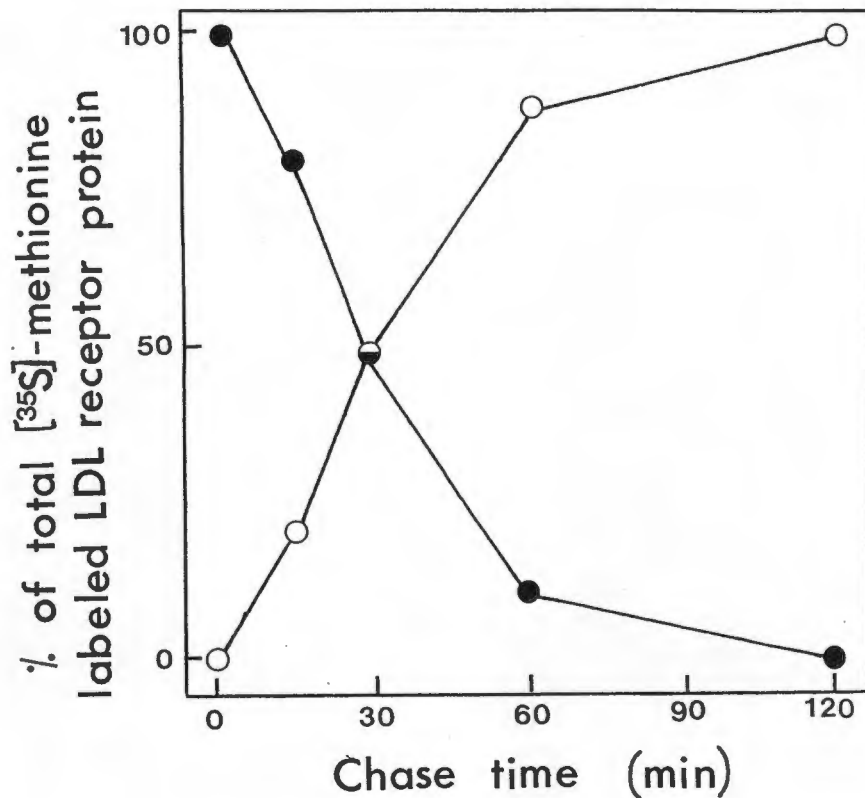


Fig 3.10: Synthesis and processing of the LDL receptor from 120 kd (●) to 160 kd (○).

HSF were grown and upregulated as described in section 3.5.1 then pulse-labeled for 30 mins with 70  $\mu$ Ci/ml of [<sup>35</sup>S]-methionine in EMEM/LPDS. The cells were chased with medium containing complete DMEM + LPDS for the indicated times, prior to being detergent solubilized. Cell extracts were incubated with preformed immune complexes containing IgG-C7, and the labeled immunoprecipitates were subjected to SDS-PAGE and fluorography. Darkened flouographic bands were extracted, and absorbances were read at 500 nm. The fraction of <sup>35</sup>S-radioactivity in each band, at each time point, was calculated by dividing the reading of the individual band by the sum of the absorbance readings of both bands at that particular chase time. Data plotted are the means of duplicates; these always differed by < 6% from each other.

30 minutes and not chased, the 120 kd band was the only immunoprecipitated band seen. After 30 minutes of chase, about 50% of the protein was converted to the 160 kd form, and by 1 hour, about 90% had been converted. As the amount of 120 kd precursor protein declined, the 160 kd protein increased correspondingly, thus the total amount of [<sup>35</sup>S]-radioactivity in both bands remained constant.

The kinetics of this conversion, and the molecular weights of the precursor and mature forms, are in agreement with the results of Tolleshaug *et al* (243) in their work describing the post-translational processing of the LDL receptor.

### 3.8.3 Downregulation of the LDL receptor.

The LDL receptors on normal human skin fibroblast monolayers were either upregulated (by incubation in DMEM/LPDS medium), or downregulated (in medium containing DMEM/LPDS supplemented with 1  $\mu$ g/ml of 25-hydroxycholesterol) for 23 hours - including the 8 hour pulse period - according to the standard procedure. After the pulse, the cells were solubilized and precipitated with immune complex containing IgG-C7 and B3/25. The fluorogram showed very distinct LDL receptor bands (160 kd) obtained from cells which had been upregulated (Fig. 3.9, lanes 1 & 2); this band was markedly decreased in precipitations which were performed on cells where the LDL receptor had been downregulated first (Fig. 3.9, lanes 5 & 6). In contrast, the amount of transferrin receptor protein which was immunoprecipitated

remained approximately constant, irrespective of whether or not the cells had been preincubated in 25-hydroxycholesterol containing medium.

#### 3.8.4 Use of another cell line.

Fibroblasts obtained from a clinically defined Xhosa patient, designated T.T., have been shown to bind  $^{125}\text{I}$ -LDL to 50-70% of normal (43), while demonstrating less than 2% binding activity of IgG-C7 at 4°C and 37°C (Coetzee et al., unpublished results). These cells, and cells from a normal HSF line, were labeled with [ $^{35}\text{S}$ ]-methionine, and immunoprecipitated with the IgG-C7 containing immune complex, according to the standard protocol. The fluorogram showed a very distinct 160 kd LDL receptor band obtained from the normal cell line (Fig. 3.9, lanes 1 & 2), while there was no detectable radioactivity in this 160 kd position using cells obtained from patient T.T. (Fig. 3.9, lanes 7 & 8). Both cell types contained transferrin receptors, which were immunoprecipitated by the B3/25 antibody present in the immune complexes. From the fluorogram, it appears that the T.T. cell line has somewhat more transferrin receptors than does the normal cell line.

The results obtained in sections 3.8.1 - 3.8.4 indicate conclusively that the 160 kd protein, which was specifically immunoprecipitated by the IgG-C7 containing immune complex, represents the mature form of the LDL receptor.

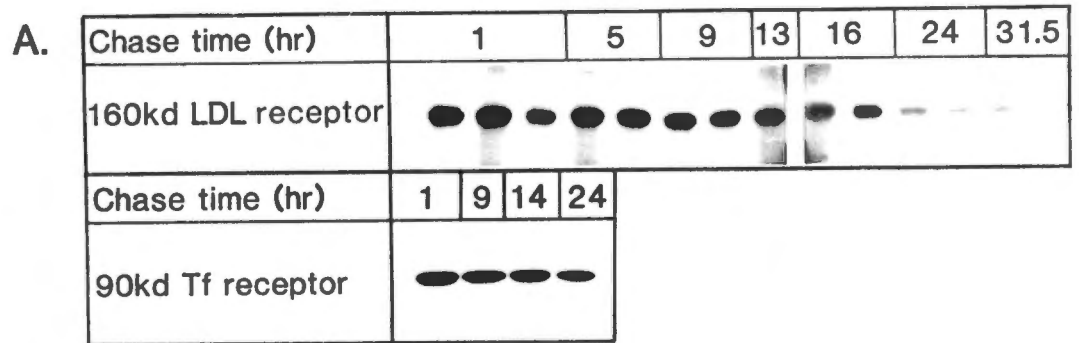
### 3.9 The half-life of the LDL (and transferrin) receptors in normal human skin fibroblasts.

The turnover rates of the LDL and transferrin receptors on a normal HSF cell line (GM 3348A), were measured using the [ $^{35}$ S]-methionine pulse-chase protocol as described in sections 3.4 - 3.7. Monolayers were incubated for 16 hours in DMEM/LPDS medium to upregulate the LDL receptors, and were subsequently pulse-labeled in methionine-free medium (EMEM) containing LPDS and [ $^{35}$ S]-methionine. The cells were then chased in complete DMEM/LPDS (containing 200  $\mu$ M unlabeled methionine) for various times before detergent solubilization. At each time, a first immunoprecipitation was performed using preformed immune complex containing IgG-C7 to precipitate the radiolabeled LDL receptor protein, and a second precipitation was done with B3/25 containing immune complex to bring down the transferrin receptors.

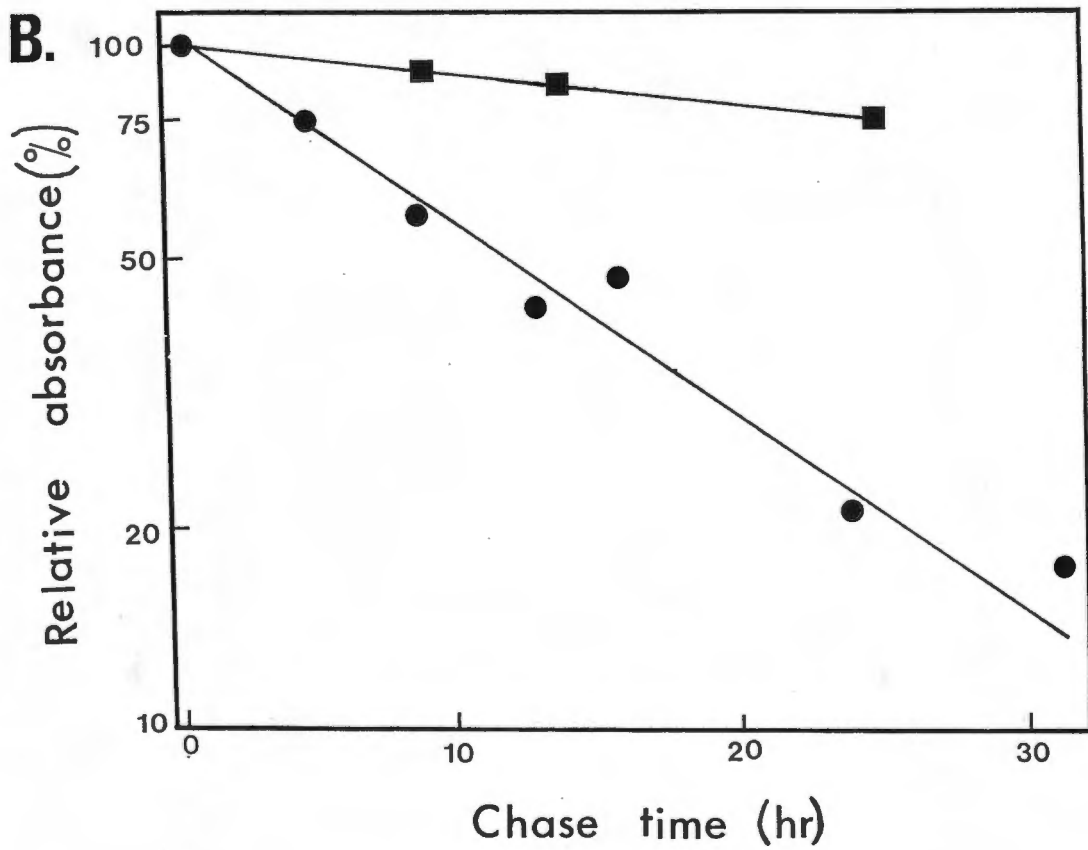
Measurement of the radioactivity remaining in the 160 kd LDL receptor bands from the fluorograms (Fig. 3.11A) yielded a typical first order decay curve with a half-life ( $t_{1/2}$ ) of  $11.7 \pm 2.2$  hr (Fig. 3.11B). These results represent the mean  $\pm$  S.D. of data obtained from 10 separate experiments. Another normal HSF line, (FG<sub>0</sub>), was examined, and a similar half-life for the LDL receptors was obtained (data not shown). Quantitation of the 90 kd transferrin receptor bands from the fluorograms (Fig. 3.11A) yielded an approximate  $t_{1/2}$  of  $55 \pm 10$  hr (Fig. 3.11B); the data depicted are one of 6 representative experiments, performed

FIGURE 3.11

A.



B.



**Fig. 3.11: Immunoprecipitation of  $^{35}\text{S}$ -labeled LDL receptors (●) and  $^{35}\text{S}$ -labeled transferrin receptors (■) from normal human skin fibroblasts.**

Monolayers were grown as described in section 3.5.1, and then incubated in DMEM/LPDS for 16 hours to upregulate the LDL receptors. The cells were pulse-labeled for 8 hours in EMEM/LPDS containing 35  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine, and then chased for the indicated times in complete medium containing 200  $\mu\text{M}$  unlabeled methionine. The detergent solubilized cell extracts were incubated with preformed immune complexes containing IgG-C7, the monoclonal antibody to the LDL receptor (●). A second immunoprecipitation with B3/25, the monoclonal antibody to the transferrin receptor, was performed on the cell extracts (■). All labeled immunoprecipitates were subjected to 5-20% SDS-PAGE and fluorography. A: Fluorograms showing the LDL- and transferrin receptors at each chase time. B: Semilog plots of the percentage absorbance (relative to the first chase point) versus chase time, were obtained after extraction of the fluorograms. LDL receptor values plotted are the means of triplicates (at the 1 hour chase-incubation time), or duplicates (at the 5, 9, 16 and 24 hour chase incubation times), or they represent single incubations (13 and 31.5 hour times). Duplicates never differed by more than 10% from the means. Similar results were obtained in 9 separate experiments. The transferrin receptor values plotted were obtained from single precipitations at each point; similar results were obtained in 5 other experiments.

separately (in several of the other experiments, longer chase-incubation times were used). This  $t_{1/2}$  value is in agreement with the published half-life of about 60 hours for these receptors on a human T cell line, which was obtained using similar procedures (173).

### 3.10 Concluding remarks.

A pulse-chase procedure, which would enable the accurate quantitation of the total cellular LDL receptors, was set up and optimized. In brief, cultured human skin fibroblasts were pulse-labeled with [ $^{35}$ S]-methionine, followed by chase incubation for various times in the presence of, excess unlabeled methionine. The monolayers were detergent solubilized, and the labeled LDL receptors in the cell extracts were specifically immunoprecipitated by incubation with preformed immune complex containing IgG-C7 (the monoclonal antibody to the ligand binding domain of the LDL receptor). Using analogous procedures, the transferrin receptors were immunoprecipitated with B3/25 (the monoclonal antibody to the transferrin receptor). From the fluorograms which were finally obtained, it was possible to visualize, and to quantitate accurately, the turnover rates of these receptors.

Using this protocol, the half-lives of LDL receptors on fibroblast monolayers were measured, in the absence of any added agents. As is shown in Fig. 3.11, the measurement of radioactivity remaining in the 160 kd LDL receptor bands from the fluorograms yielded a typical first order decay

curve, with a  $t_{1/2}$  of  $11.7 \pm 2.2$  hr (mean  $\pm$  S.D. of data obtained from 10 separate experiments). Very recently, Knight et al (128) reported a  $t_{1/2}$  of 12 hr for the LDL receptor on the same cell type, using a similar approach. This half-life of about 12 hours is markedly shorter than that obtained in 1975 by Brown and Goldstein (32) for these receptors. Their value of 25-30 hours was determined by performing a  $^{125}\text{I}$ -LDL binding assay on intact fibroblast monolayers, which had been incubated for various times in the presence of the protein synthesis inhibitor, cycloheximide.

The results depicted in Fig. 3.11 show that the transferrin receptors turned over much slower than the LDL receptors in cultured HSF. The turnover rates of other plasma membrane receptors have been well documented in the literature; it appears that most have half-lives between 10 and 80 hours (section 3.1). Thus, it would seem that receptor proteins are not turned over as a unit with the plasma membrane; rather, it appears probable that there may be some mechanism(s) which determine the unique rate at which each of the plasma membrane receptor types is turned over.

In the following section, an in-depth study of LDL receptor turnover is presented, including implications as to LDL receptor up- and downregulation, as well as the effect(s) of various agents - such as cycloheximide - on the half-life. Possible site(s) and mechanism(s) of degradation of the LDL receptors were also investigated using this pulse-chase protocol, in an attempt to gain insight into the factors,

(other than synthesis), governing the expression of this important transmembrane lipoprotein receptor.

## SECTION FOUR

### CELLULAR LDL RECEPTOR DEGRADATION.

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#### 4.1 Introduction.

Although the process of receptor-mediated endocytosis has been extensively studied, and much is known about the synthesis and processing of LDL receptor molecules, the intracellular site(s) and biochemical mechanism(s) responsible for the final degradation of LDL receptors have not been elucidated (reviewed in 34, 88, 247). It has been shown that receptor degradation and ligand breakdown are not necessarily coupled events; there are many well documented cases where receptors have been observed to recycle in bulk to the cell surface subsequent to internalization, while some, or all, of their ligands are degraded in lysosomes. Thus, asialoglycoprotein receptors in isolated rat hepatocytes recycle many times in this manner, while their endocytosed ligand is degraded (14, 225); LDL receptors (88) and insulin receptors (135, 153) also escape degradation and are recycled. Transferrin receptors repeatedly traverse the endocytotic pathway together with bound protein ligand (262). In contrast, some receptors, such as those specific for EGF in HSF (55, 65), are degraded together with their ligand subsequent to internalization.

A half-life of  $11.7 \pm 2.2$  hours was obtained for the LDL receptor on cultured HSF, using the radiolabel pulse-chase procedure as described in Section Three. This was significantly shorter than that obtained by Brown and Goldstein in 1975 (32): their assay of the decreased surface ligand-binding activity after the administration of the protein synthesis inhibitor, cycloheximide, yielded a

$t_{1/2}$  of 25-30 hours. This agent, however, inhibits general cellular proteolysis as well as protein synthesis (81). Knutson et al (131), using the heavy-isotope density shift technique, showed that cycloheximide inhibited not only the synthesis of new insulin receptors but also the inactivation of existing ones in mouse fibroblast 3T3-C2 cells. Similar effects were observed in the presence of puromycin, patamycin and actinomycin D. These authors concluded that inactivation of the insulin receptor was mediated by a rapidly turning over protein (the mRNA of which was also short-lived).

The possibility thus exists that determination of the half-life of the LDL receptor in the presence of cycloheximide leads to an overestimation of the actual value. It is noteworthy in this connection that the addition of 25-hydroxycholesterol to fibroblasts produces downregulation of the receptor number which occurs significantly faster ( $t_{1/2} = 10$  hr) than when the same effect is produced in the presence of cycloheximide (32). Van der Westhuyzen and Coetzee (247) have suggested that the hydroxycholesterol could cause an increased rate of receptor degradation. This is plausible, since Tanaka et al (235) have shown that the rapid and very marked decrease in HMG CoA reductase activity which is caused by the addition of 25-hydroxycholesterol is due less to a decreased rate of synthesis than to a dramatic increase in the rate of enzyme degradation. Gil et al (78) subsequently showed that the truncated soluble part of the HMG CoA reductase protein has full enzymatic activity, while the membrane-bound domain plays a crucial role in the rapid,

regulated degradation of the enzyme protein as a whole. It is also worth noting that Ronnett et al (195), using fully differentiated 3T3-L1 adipocytes, found no change in the rate of insulin receptor synthesis associated with up- or downregulation of receptor number, but they noted that the receptor half-life increased from 8.1 to 14.8 hr under upregulation conditions. A similar effect was reported by Kasuga et al (120), who established that insulin-induced receptor loss in cultured lymphocytes was indeed caused by accelerated degradation of the receptor.

There are two general classes of mechanisms by which protein degradation occurs in living cells: one is partly or entirely lysosomal, while another is independent of lysosomes (101). Proteolytic inhibitors such as leupeptin (168) and lysosomotropic agents such as chloroquine (54) and ammonium chloride (54) are often used, somewhat crudely, to distinguish between lysosomal and non-lysosomal degradative pathways. Furthermore, incubation of cells at 18°C, a condition in which lysosomal delivery of intracellular vesicular material is completely inhibited (202), permits an assessment of the possible contribution of these organelles to a particular degradation process.

In the study reported herein, the pulse-chase protocol, as described in Section Three, was used to investigate whether cycloheximide exerts an inhibitory effect on the degradation of the LDL receptor; such a finding would indicate that the presently accepted value of the half-life of this receptor may be an overestimate. The effect(s) of LDL and 25-

hydroxycholesterol on the receptor turnover rate were also examined. Furthermore, the up- and downregulation kinetics of the LDL receptor (as measured by the binding of  $^{125}\text{I}$ -LDL to fibroblast monolayers) were compared, to assess whether the receptor degradation rate is altered during these changes in physiological status of the cultured cells. In the case of cell surface receptors, it is unknown whether receptor degradation is governed by the susceptibility of the molecules to particular proteases, or by the rate at which they are segregated into a defined degradation compartment. The turnover rate of LDL receptors in an internalization defective fibroblast line, GM 2408A (J.D.), was examined in order to ascertain whether internalization, clustering or recycling of LDL receptors are prerequisites for their eventual degradation. The possible mechanism(s) involved in the degradation of the LDL receptor were also investigated using (i) agents known to be lysosomotropic, or (ii) altered chase-incubation temperatures. Finally, the susceptibility to degradation of desialylated receptors (obtained from cells which had been neuraminidase treated subsequent to pulse-labeling), was compared with that of normal receptors.

#### 4.2 Experimental procedures.

Unless otherwise specified, all materials and methods used were as described in previous sections, or in the Appendix.

#### 4.2.1 Stock solutions.

Stock solutions were made up as follows:

25-hydroxycholesterol: 0.5 mg/ml in ethanol; always vortexed into the incubation medium to give a final concentration of 1  $\mu$ g/ml.

Cycloheximide: 10 mM in PBS, used at a final concentration of 100  $\mu$ M.

Ammonium chloride: 1 M in PBS, used at a final concentration of 10 mM.

Leupeptin: 15 mg/ml in H<sub>2</sub>O, used at 150  $\mu$ g/ml finally.

Chloroquine: 2 mM in DMEM, always made up immediately prior to the experiment, and used at a final concentration of 70  $\mu$ M.

Monensin: 10 mM in ethanol, vortexed into the incubation medium to yield an eventual concentration of 25  $\mu$ M.

#### 4.2.2 Cells

The human skin fibroblast strains GM 3348A (LDL receptor normal) and GM 2408A (LDL receptor internalization defective), were obtained from the Human Genetic Cell Repository (Camden, N.J., USA), and were maintained in culture as described in section 2.2.1 (for intact cell assays) or section 3.5.1 (for pulse-chase experiments). The human hepatoma cell line, Hep G2, was a gift from Dr. Barbara Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania, USA). They were cultured for pulse-chase experiments as follows:  $4.3 \times 10^6$  Hep G2 cells

were seeded per 60 mm petri dish in MEM supplemented with 10% fetal calf serum, and on day 2 of cell growth, upregulation of the LDL receptors was performed as described for cultured fibroblasts (section 3.5.1). Human lymphoblastoid cells were obtained and cultured as described in section 2.2.1.

#### 4.2.3 Preformed immune complexes

Immune complexes were made as described in section 3.4.1. They contained goat anti-mouse IgG and one, or a combination of (see legends to figures), the following mouse monoclonal antibodies: IgG-C7 or B3/25. Immune complexes were also prepared using goat anti-rabbit IgG, and one of the following rabbit antibodies: polyclonal antibody to the LDL receptor, or rabbit nonimmune IgG.

Pulse-chases and immunoprecipitations of  $^{35}\text{S}$ -labeled LDL and transferrin receptors were performed as described in section 3.6. The precipitation of transferrin receptors was used as a means of assessing whether the effect(s), if any, noted under the various experimental conditions were specific to the LDL receptor only, and, occasionally (see Fig. 4.10), to compensate for differential losses incurred during receptor isolation.

#### 4.2.4 Assays to assess $^{125}\text{I}$ -LDL binding and metabolism.

Surface binding of  $^{125}\text{I}$ -LDL at 4°C - Fibroblast monolayers which had been incubated for 48 hours in DMEM/LPDS to upregulate the LDL receptors, were chilled to 4°C and washed once with ice-cold PBS.  $^{125}\text{I}$ -LDL binding was measured, using 10  $\mu\text{g/ml}$   $^{125}\text{I}$ -LDL either in the absence or in the presence of 200  $\mu\text{g/ml}$  unlabeled LDL (15). High-affinity binding was determined by subtracting the values for  $^{125}\text{I}$ -LDL bound in the presence of excess unlabeled ligand (non-specific binding) from that bound in its absence (total binding).

Surface binding assay to assess LDL receptor redistribution after incubation with various agents - Upregulated fibroblast monolayers were incubated with 3 ml/dish fresh DMEM/LPDS + 40  $\mu\text{g/ml}$  LDL, supplemented with the appropriate agents, as detailed in the legend to Table 4.2. After either 1 hr or 5 hr at 37°C, the surface-bound, unlabeled LDL was removed by treatment with 0.4% heparin/PBS for 40 mins at 4°C, and  $^{125}\text{I}$ -LDL binding was subsequently effected for 1 hr at 4°C as described above.

Metabolism of  $^{125}\text{I}$ -LDL after incubation at 37°C - The amounts of surface-bound, intracellular and degraded  $^{125}\text{I}$ -LDL, after incubation of fibroblasts at 37°C for 4 hr, were measured as described in section 2.2.6.

4.2.5 The effect of 25-hydroxycholesterol on the basal intracellular proteolysis of long- and short-lived proteins in normal lymphoblastoid cells.

Normal lymphoblastoid cell cultures were grown for 43 hours at  $1 \times 10^6$  cells/ml in growth medium (MEM + 10% HIFCS) containing 1  $\mu\text{g/ml}$  [ $^3\text{H}$ ]-phenylalanine. After 30 hours, an additional volume of medium was added to maintain an approximately constant cell density. This procedure - Method A - resulted in the preferential labeling of proteins having relatively long half-lives. To label short-lived proteins (Method B), other normal lymphoblastoid cell cultures were maintained in full growth medium containing 4  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-phenylalanine for 1 hour. The analysis of basal intracellular proteolysis was then performed, in either case, as follows: the cells were spun briefly (2 000 rpm, 1 min, 4°C) to remove the radioactive medium, and were then washed twice (as above) with ice-cold medium (MEM + 10% HIFCS) to remove all extracellular [ $^3\text{H}$ ]-phenylalanine. To reduce the large intracellular acid-soluble pool of radioactivity to an acceptable background level before the net release of radioactivity from intracellular labeled protein could be measured, the cells were incubated under conditions allowing carrier-mediated transport of [ $^3\text{H}$ ]-phenylalanine out of the cells. In Method A, this entailed four consecutive incubations at 37°C, each in fresh growth medium with cell density at  $1 \times 10^6$  cells/ml: the first, second and fourth incubations were for 30 mins each, while the third was for 16 hours. In Method B, the washing out of the intracellular soluble

radioactivity was carried out by performing three consecutive washes at 37°C (each at  $1 \times 10^6$  cells/ml) for 20, 40 and 60 minutes (first, second and third washes, respectively). For both methods, the cells were finally resuspended at  $1 \times 10^6$  cells/ml in fresh growth medium containing 1 mM excess non-labeled phenylalanine, in the absence or presence of 0.6  $\mu$ g/ml 25-hydroxycholesterol; the release of labeled amino acid into this medium was used to measure intracellular proteolysis. Cells were incubated in the various media at 37°C for the indicated times. Thereafter, the medium was removed by brief centrifugation of the cell suspensions (2 000 rpm, 1 min, 4°C), and assayed for TCA-soluble radioactivity by precipitation with an equal volume of 20% TCA for 1 hour at 4°C. The cell pellets were washed twice with PBS (4°C) by centrifugation as described above; 1 ml 1% SDS was then added to solubilize each cell pellet. These lysates were briefly sonicated, a 500  $\mu$ l aliquot was counted to determine the total radioactivity within the cells, and the remaining 500  $\mu$ l was supplemented with 2 mg BSA as carrier, precipitated with an equal volume of 20% TCA at 0°C for 1 hour and then spun at 2 000 rpm for 15 mins. The supernatant was counted to obtain the residual amount of intracellular TCA-soluble radioactivity. All counting procedures were performed in a Beckman scintillation counter using Beckman Ready-Solv EP as scintillation fluid. Intracellular protein degradation in each case was expressed as a % of the initial total protein radioactivity that was released into the chase medium ie. % proteolysis = (dpm [ $^3$ H]-phenylalanine in TCA-soluble medium)/(dpm in medium + dpm in TCA precipitable cell fraction).

#### 4.2.6 Other Assays

In the cases where cells were incubated in the presence of 1  $\mu\text{g/ml}$  25-hydroxycholesterol, the amount of [ $^{35}\text{S}$ ]-methionine incorporated into total cell protein was measured (TCA precipitation of protein). No significant inhibition of the rates of total cell protein synthesis was obtained.

The inhibition of protein synthesis with cycloheximide was assayed by measuring the amount of [ $^3\text{H}$ ]-phenylalanine incorporated into total cell protein. After 1 hr, protein synthesis was inhibited by 98%, while the cells remained >95% viable, as determined by trypan blue dye exclusion. Cell protein was measured by the Lowry method (147) using BSA as a standard.

Unless otherwise specified, all [ $^{35}\text{S}$ ]-methionine pulse-chase experiments were performed on the LDL receptor normal human skin fibroblast cell line, GM 3348A.

Where relevant, statistical analysis of data was performed. Best fit curves were obtained using a weighted non-linear regression programme adapted from Duggelby (64), and the parameters thus derived were compared using Peritz' F test (95).

### 4.3 Results

#### 4.3.1 The LDL receptor half-life remains constant over a range of cellular receptor protein content.

Using the [<sup>35</sup>S]-methionine pulse-chase protocol, the LDL receptors on HSF were found to turn over with a  $t_{1/2}$  of  $11.7 \pm 2.2$  hr (section 3.9). This result was obtained after an upregulation period of 16 hr, followed by an 8 hr pulse-label in EMEM/LPDS i.e. a 24 hr upregulation prior to initiating the chase incubation (at this 24 hr time, cell-surface LDL receptor expression was about 85% of the maximum value - see Fig. 4.2). To ascertain whether this half-life remained constant in the fully upregulated or in the downregulating states of the receptors, other monolayers were incubated in DMEM/LPDS for 40 hr before initiating an 8 hr pulse (i.e. >97% of maximal cell-surface LDL receptor expression at the end of the pulse-incubation), and then chased for varying times in DMEM/LPDS. Under these conditions of maximal LDL receptor upregulation at the start of the chase (i.e. no further increase in receptor number during the chase), the half-life of the receptors remained unchanged ( $P > 0.05$ ) at  $t_{1/2} = 11.5$  hr (Fig. 4.1). Alternately, monolayers were maintained in full growth medium (DMEM supplemented with 10% HIFCS), resulting in a low level of LDL receptor expression. After pulse-labeling for 8 hr in EMEM/LPDS (i.e. about 45% of maximal LDL receptor expression induced by the end of the pulse-period), the cells were chased in the upregulation medium supplemented with 10  $\mu$ g/ml LDL, to suppress any further increase in LDL

FIGURE 4.1

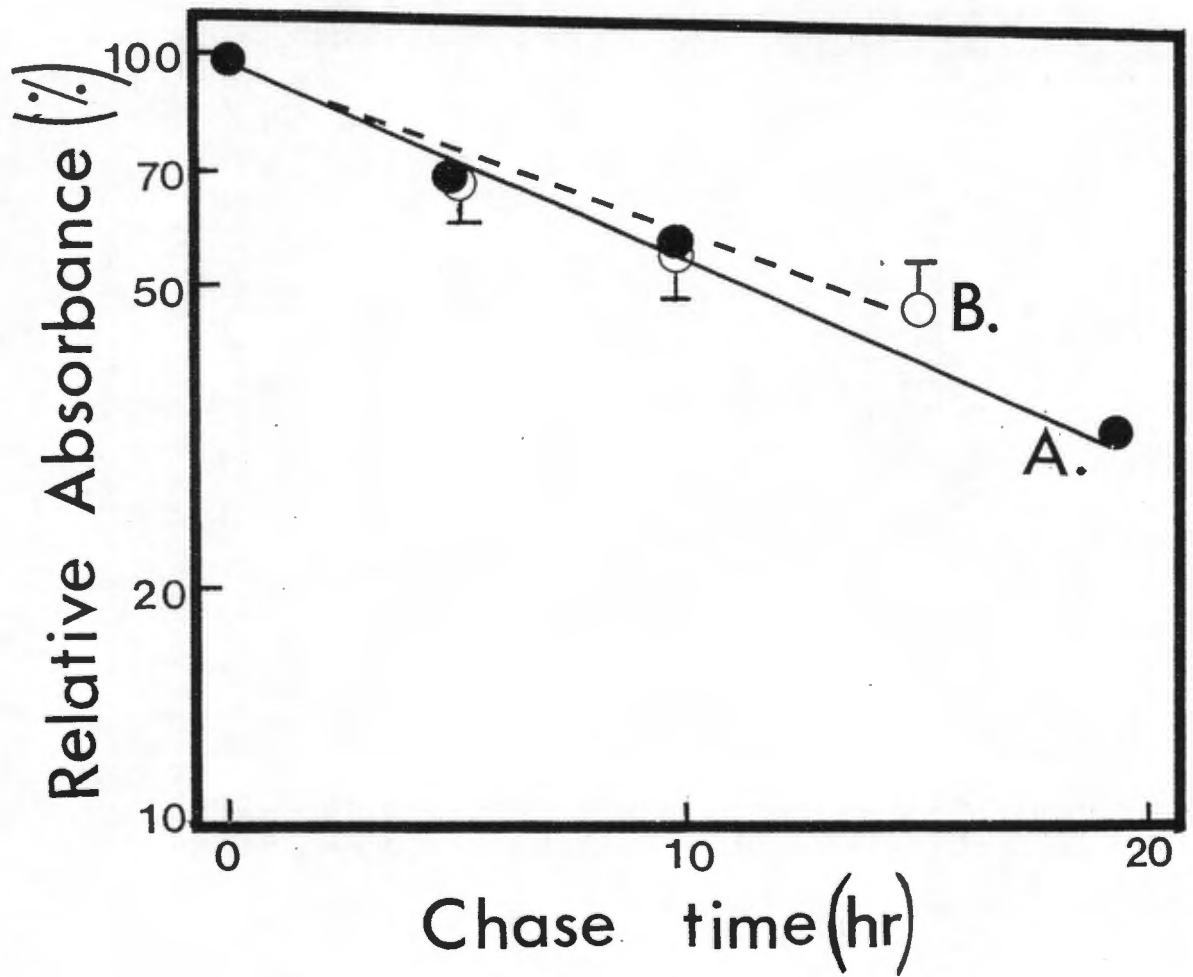


Fig. 4.1: Turnover of  $^{35}\text{S}$ -labeled LDL receptors from normal fibroblasts.

In Procedure A (●, solid line) the monolayers were upregulated for 40 hr, then pulse-labeled for 8 hr with EMEM/LPDS containing 35  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine. Chase-incubations were performed for the indicated times in DMEM/LPDS (containing 200  $\mu\text{M}$  methione). In Procedure B (○, dashed line) the monolayers were maintained in DMEM supplemented with 10% HIFCS. 30 mins prior to initiating the pulse, the cells were incubated with DMEM/LPDS at 37°C. The cells were then pulse-labeled in EMEM/LPDS containing 100  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine for 8 hr, and chase-incubated for the indicated times in complete DMEM/LPDS supplemented with 10  $\mu\text{g/ml}$  LDL. In both procedures, immunoprecipitation and quantitation of labeled LDL receptors were performed as described in "Experimental Procedures". Semilog plots are shown, depicting the percentage absorbance (relative to the first chase point) versus chase time. Values plotted as (●) are the means of duplicates obtained from a single experiment; duplicates never differed by more than 10% from the means. The values plotted as (○) represent the mean  $\pm$  S.D. of results obtained from triplicate precipitations at each time in a single experiment.

receptor number. As shown in Fig. 4.1, the half-life of the LDL receptors under these downregulating conditions was not significantly different ( $P > 0.05$ ) from that obtained using fully upregulated cells ( $t_{1/2} = 13.7$  hr). In the downregulating procedure, shorter chase times were used, since little radioactive label was incorporated into the LDL receptors under these conditions, resulting in fluorogram bands which were too faint if longer chase times were used. Thus, the half-lives of the LDL receptors remained unchanged over the range of cellular LDL receptor content tested.

#### 4.3.2 A comparison of the up- and downregulation kinetics of LDL receptors.

The normal downregulation kinetics of LDL receptors in fibroblasts were measured by adding medium containing 40  $\mu\text{g/ml}$  LDL to previously fully upregulated monolayers (Fig. 4.2A). The surface LDL receptors were measured at various times by  $^{125}\text{I}$ -LDL binding at 4°C. The receptor activity after addition of LDL remained constant for about 4-5 hr before it began to decrease with apparent first order kinetics. The apparent half-life determined from this procedure (using the end of the lag phase as the starting point) was 13 hr, similar to that found above (section 4.3.1). The upregulation curve was a mirror image of the downregulation curve (excluding the lag phase). This confirms that up- and downregulation are largely due to changes in the receptor synthetic rates. First order kinetic curves using a  $t_{1/2} = 11.7 \pm 2.2$  hr were generated

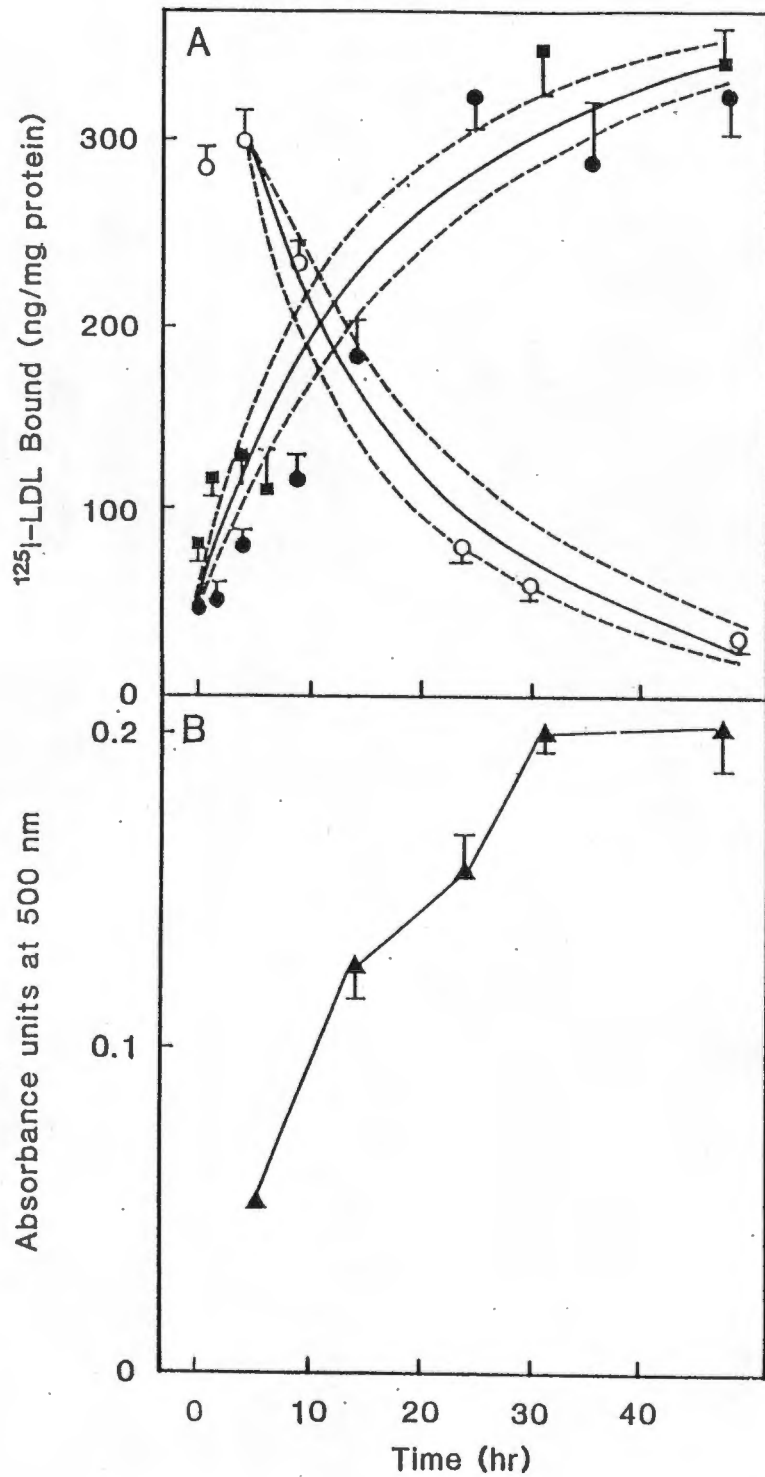


Fig. 4.2: A comparison of the upregulation (●, ■, ▲) and downregulation (○) kinetics of LDL receptors in fibroblast monolayers.

A: In the upregulation protocol, monolayers were incubated in DMEM/LPDS for the indicated times (Note: No prior upregulation of LDL receptors). For the downregulation kinetics, cells were first incubated for 48 hr in medium containing

LPDS, after which each monolayer received fresh LPDS medium, supplemented with 40  $\mu\text{g/ml}$  LDL. The cells were then maintained at 37°C for the indicated times, after which they were further incubated with fresh DMEM/LPDS medium (containing no added lipoproteins) for 30 mins at 37°C. (The latter incubation ensured that no LDL occupied surface receptors in the case of the "downregulation protocol"). The monolayers were then chilled to 4°C, and high-affinity binding (in the case of both types of receptor regulation), was measured as described in section 4.2.4. Results were calculated from data obtained from triplicate dishes at each point. Non-specific binding was always <10% of total binding in the fully upregulated state. All three experiments ( $\bullet$ ,  $\blacksquare$ ,  $\circ$ ) were performed on different days, using different batches of  $^{125}\text{I}$ -LDL in each case. Theoretical LDL receptor up- and down- regulation kinetics were obtained using the first order exponential equation; in each case, the solid line represents the kinetics obtained using an LDL receptor half-life = 11.7 hr, with the broken lines representing  $\pm$  one standard deviation from this.

B: Fibroblast monolayers, which had been maintained in growth medium (DMEM + 10% HIFCS), were washed with PBS, and then incubated in DMEM/LPDS to upregulate LDL receptors. 30 mins before each of the indicated times, this medium was replaced with EMEM/LPDS, and each dish was subsequently pulse-labeled with 60  $\mu\text{Ci/ml}$  of [ $^{35}\text{S}$ ]-methionine for 90 mins. Immunoprecipitation of labeled LDL receptors was then performed as described in "Experimental Procedures". Results represent the mean  $\pm$  S.D. of data obtained from triplicate dishes at each point.

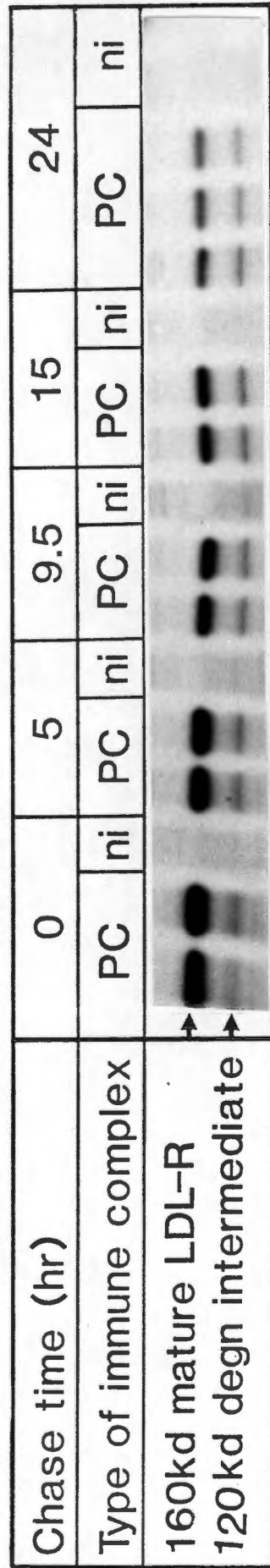
and also plotted for comparative purposes. These curves were remarkably similar to those obtained for the up- and downregulation kinetics.

The synthesis rates of mature LDL receptors in fibroblasts which were incubated for various times in LPDS medium, were measured by pulse-labeling the receptors for 1.5 hr at each of the time points. As is depicted in Fig. 4.2B, the LDL receptor synthesis rate increased linearly up to 30 hr, after which it became constant at this high level.

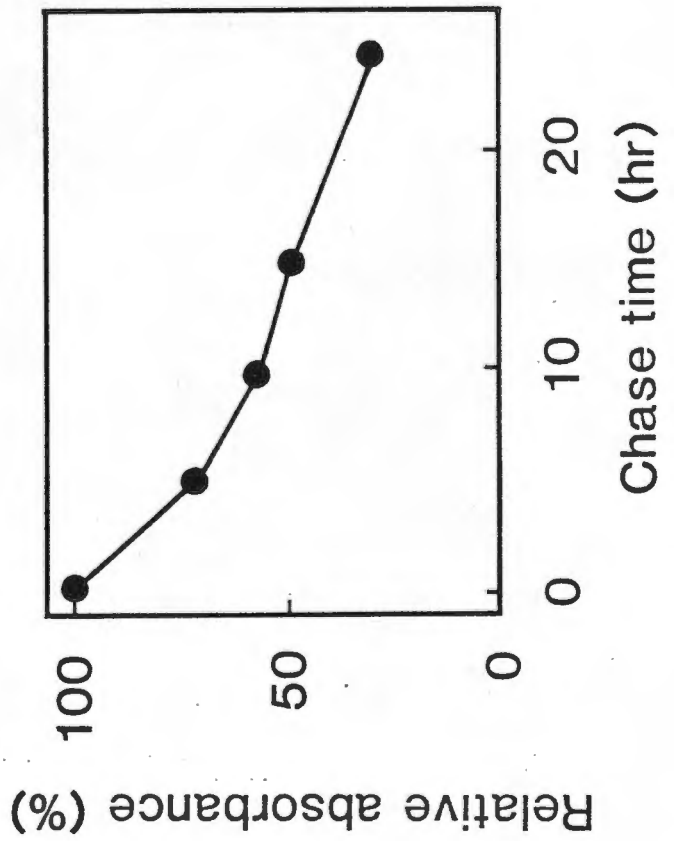
#### 4.3.3 A putative degradation intermediate of the LDL receptor.

Lehrman et al (140) have observed a molecule that is apparently a high molecular weight intermediate of LDL receptor degradation in cultured HSF, and similar observations were made by Kozarsky et al (133) in the case of Chinese Hamster Ovary cells. In this study, such intermediates were sought in pulse-labeled cell extracts subjected to immunoprecipitation with a rabbit polyclonal antibody directed at the entire LDL receptor. Two labeled peptides with apparent molecular weights of 160 kd and approximately 120 kd were immunoprecipitated with the polyclonal antibody at each time point (Fig. 4.3A). Immunoprecipitations were also performed using control nonimmune IgG, and in no case was either of these proteins detected. The 160 kd band clearly represented the mature LDL receptor; it had a half-life of 13.7 hr, which was not

**FIGURE 4.3**



A.



B.

**Fig. 4.3: Degradation of  $^{35}\text{S}$ -labeled LDL receptors and the formation of a putative degradation intermediate from normal fibroblasts.**

Monolayers were pulse-labeled for 8 hr in EMEM/LPDS containing 70  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine, then chase-incubated for the indicated times in complete DMEM/LPDS medium. Cell extracts were incubated with preformed immune complexes containing either rabbit polyclonal antibody (PC), or rabbit non-immune IgG (ni). The  $^{35}\text{S}$ -labeled immunoprecipitates were subjected to 5-20% SDS-PAGE and fluorography. Precipitations with the polyclonal antibody were performed in duplicate, after chase-incubation times of 0, 5, 9.5 and 15 hours (values never differed by more than 7% from the means), or in triplicate, after chase-incubating for 24 hours; those with the non-immune IgG were done using single dishes. The data represent results obtained from a single experiment. (A) Fluorogram showing the mature 160 kd LDL receptor and the putative 120 kd LDL receptor degradation intermediates precipitated with the polyclonal antibody, at various chase times. The non-immune IgG precipitated neither of these proteins. (B) Plot of the relative percentage absorbance of the 160 kd LDL receptor (expressed relative to the 160 kd LDL receptor at zero hr) at each chase time.

degn = degradation

significantly different ( $P > 0.05$ ) from that of the LDL receptor when the latter was immunoprecipitated with IgG-C7 (Fig. 4.3B). The 120 kd band remained virtually unchanged in absolute amount throughout the chase period (Fig. 4.3A). It thus seems likely that the 120 kd protein was a degradation product of the mature 160 kd form of the LDL receptor, analagous to the degraded forms of the LDL receptor mentioned above. The label detected in the 120 kd band at zero chase time could have been present either in the known precursor of mature LDL receptors (about 120 kd) or the degradation fragment with its very similar molecular weight on SDS-PAGE analysis under reducing conditions. At later chase time points, however, the label clearly was not due to the receptor precursor, since all of it had been converted to the 160 kd mature form within 1 hour after a 30 minute pulse-label period in other experiments (data not shown). If the 120 kd protein was in fact an intermediate in the LDL receptor degradation pathway, a constant flux through this intermediate form must have occurred, followed by a slow proteolytic step(s) catabolizing the large fragment. A similar situation has previously been observed in the case of apolipoprotein B degradation as catalysed by cathepsins in the lysosomes of cultured cells (11, 249).

#### **4.3.4 The turnover of LDL receptors in two other cell types.**

A second human cell type, Hep G2 cells, representing well-differentiated hepatoma cells, was also investigated with

respect to the turnover rates of both LDL and transferrin receptors (Fig. 4.4). A half-life of 8.5 hr was obtained in the case of LDL receptors, using the procedure outlined in Fig. 3.11; this was not significantly different ( $P > 0.05$ ) from that obtained for fibroblasts. Transferrin receptors in Hep G2 cells turned over with a half-life of 12.5 hr; this was markedly less than the value of 55 hours observed for this receptor in fibroblasts (Fig. 3.11), and the value of 60 hours obtained by Omary and Trowbridge (173) in a human leukemic T-cell line.

In addition, the expression of LDL receptors on cultured human lymphoblastoid cells was measured by assaying the cell-associated  $^{125}\text{I}$ -LDL on intact cells (Fig. 4.5) (it was not possible to perform the pulse-chase experiments using IgG-C7 to immunoprecipitate LDL receptors, since lymphoblastoid cells contain Fc receptors which bind to the monoclonal antibodies). The LDL receptors were upregulated prior to commencing the experiment, and the cells were then incubated for the indicated times in medium supplemented with the agents as described in the figure legend. Assays were also performed to check (i) that cycloheximide inhibited general protein synthesis  $> 95\%$ , while cells remained more than 90% viable, as determined by trypan blue dye exclusion, and (ii) that the presence of the compounds did not alter the efficiency of internalization of the LDL receptors (data not shown). That the internalization indices were unaltered, indicated that the decreased  $^{125}\text{I}$ -LDL uptake values obtained were due to a decrease in binding to the LDL receptor, and not to a reduction in the

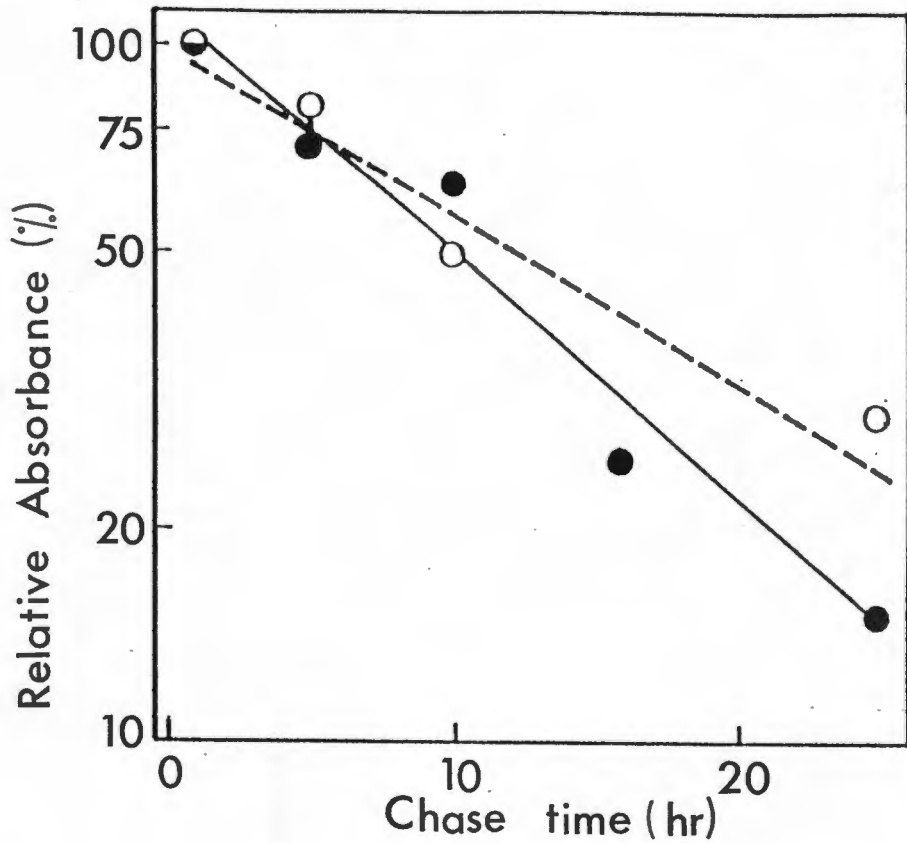
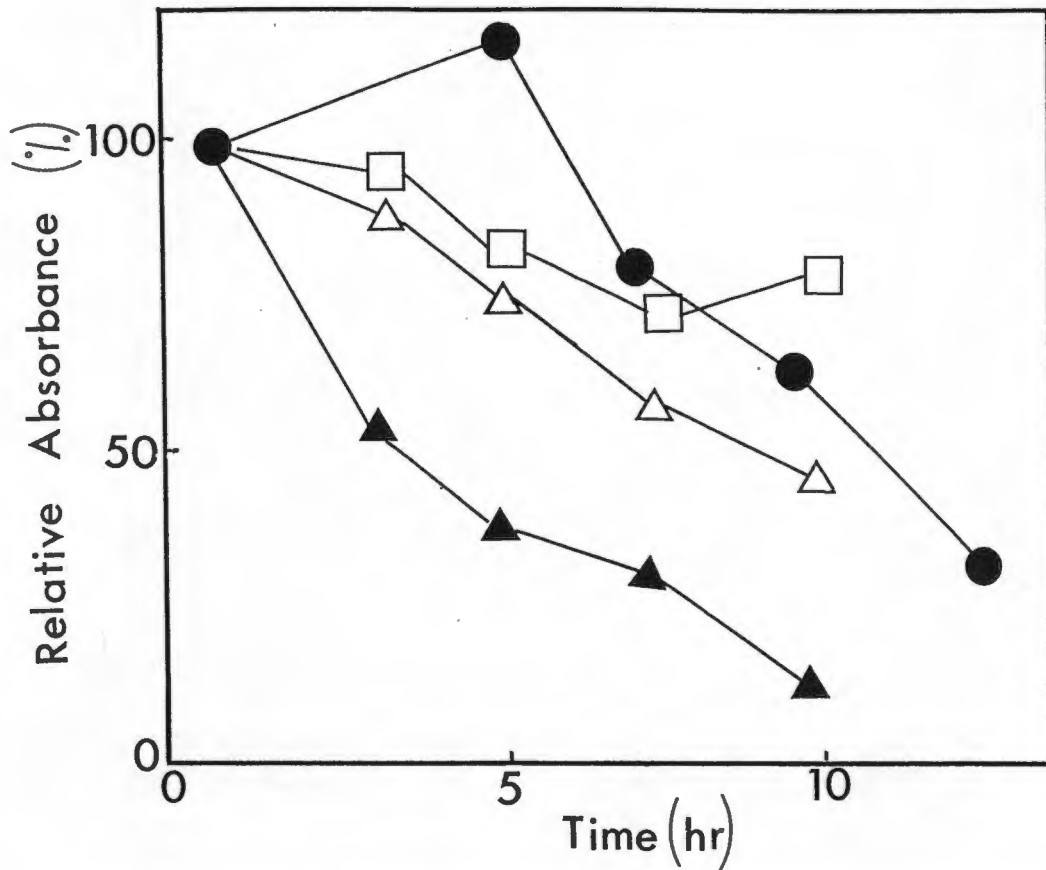


Fig. 4.4: Turnover of  $^{35}\text{S}$ -labeled LDL receptors and  $^{35}\text{S}$ -labeled transferrin receptors from cultured Hep G2 cells. Monolayers were incubated for 16 hr with DMEM/LPDS, then pulse-labeled for 8 hr with  $50 \mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine. The monolayers were chase-incubated for the indicated times, after which the detergent solubilized cell extracts were incubated (first precipitation) with preformed immune complexes containing IgG-C7, (●, solid line), followed by a second immunoprecipitation, using immune complexes containing B3/25 (○, dashed line). The  $^{35}\text{S}$ -labeled immunoprecipitates were subjected to 5-20% SDS-PAGE and fluorography. Semilog plots of the percentage absorbance (relative to the first chase point) versus time of chase were constructed. Values plotted represent the mean of results obtained from duplicate (●) or single (○) precipitations at each point in a single experiment. Duplicates never differed by more than 10% from the means.



**Fig. 4.5: Surface LDL receptors in lymphoblastoid cells, as assayed after the administration of various agents.**

Lymphoblastoid cells were incubated in LPDS medium for 24 hr as described in section 2.2.5. Incubations were then performed at 37°C for the indicated times in MEM, supplemented with 10% HIFCS and 40  $\mu$ /ml LDL, in the presence of: no further additions (●), 30  $\mu$ M cycloheximide (□), 0.6  $\mu$ g/ml 25-hydroxycholesterol (▲), or cycloheximide + 25-hydroxycholesterol (△). The cells were then maintained for a further 30 mins at 37°C in LPDS containing medium to remove bound LDL. Cells were further incubated in medium containing  $^{125}$ I-LDL (10  $\mu$ g/ml)  $\pm$  LDL (300  $\mu$ g/ml) for 30 mins at 37°C. High-affinity cell-associated values were calculated as described in section 2.2.7. Results represent cell-associated  $^{125}$ I-LDL values expressed as a % of a control (no additions, assayed at zero time). All the data plotted were obtained in single experiments, and they typify the results obtained in 3 separate experiments.

internalization of bound LDL (Note: no degradation of the ligand occurred after incubating for 30 minutes at 37°C - section 2.2.7). Incubation in the presence of LDL only, or LDL + 25-hydroxycholesterol, resulted in a loss of surface receptors, with first order kinetics (a lag phase of about 5 hours was noted in the presence of LDL only, before any decrease was observed); the half-life of the LDL receptors was estimated to be about 4 hours in each of these cases. Cycloheximide greatly reduced the rate of measured receptor loss, a trend which was also noted when cycloheximide and 25-hydroxycholesterol were added simultaneously.

In section 2.3.3, an average LDL receptor recycling time of about 10.5 minutes was calculated, using data obtained from 4 hour, 37°C incubations, and assuming that receptor downregulation (turnover) could be ignored during such an incubation. The current results indicate that this assumption is valid, since effective downregulation of LDL receptors only became apparent after a lag phase of 5 hr.

#### 4.3.5 The turnover of LDL receptors in human skin fibroblasts in the presence of various agents.

Since LDL, 25-hydroxycholesterol and cycloheximide differentially affected LDL receptor turnover in lymphoblastoid cells, the effects of these agents in HSF were analysed as well. It is known that these agents alter the cell surface expression of the LDL receptor in HSF (32). Thus, the turnover of LDL receptors was measured in this

cell type as before (ie. [<sup>35</sup>S]-methionine pulse-chase) in the presence of: 50  $\mu$ g/ml LDL (to check for possible ligand-induced changes in receptor turnover); 1  $\mu$ g/ml of 25-hydroxycholesterol (which causes a marked decrease in LDL receptor expression after uptake of the sterol by non-receptor-mediated processes); 100  $\mu$ M cycloheximide (at a concentration giving almost total inhibition of protein synthesis in the cells). In the case of the first two agents, the viability of cells after chase-incubations was checked by assaying the rates of total protein synthesis, which were the same as in the untreated controls (data not shown), indicating that any effect(s) produced by these agents were not due to toxicity and decreased cell viability. As shown in Fig. 4.6, the LDL receptor half-life obtained after chase-incubating in the presence of LDL or 25-hydroxycholesterol did not differ significantly ( $P > 0.05$ ) from that obtained in the absence of the added agents. The presence of cycloheximide in the chase-medium produced an initial transient decrease in the number of LDL receptors, roughly similar to that obtained in the absence or presence of the other agents. After about 5 hr, however, the rate of receptor loss decreased markedly, resulting in an apparently constant content of labeled receptors over the next 20 hr. The addition of either 25-hydroxycholesterol or cycloheximide to the chase media produced no marked effect on the turnover rate of transferrin receptors; in both instances, approximately 25% of these receptors were degraded after 25 hr.

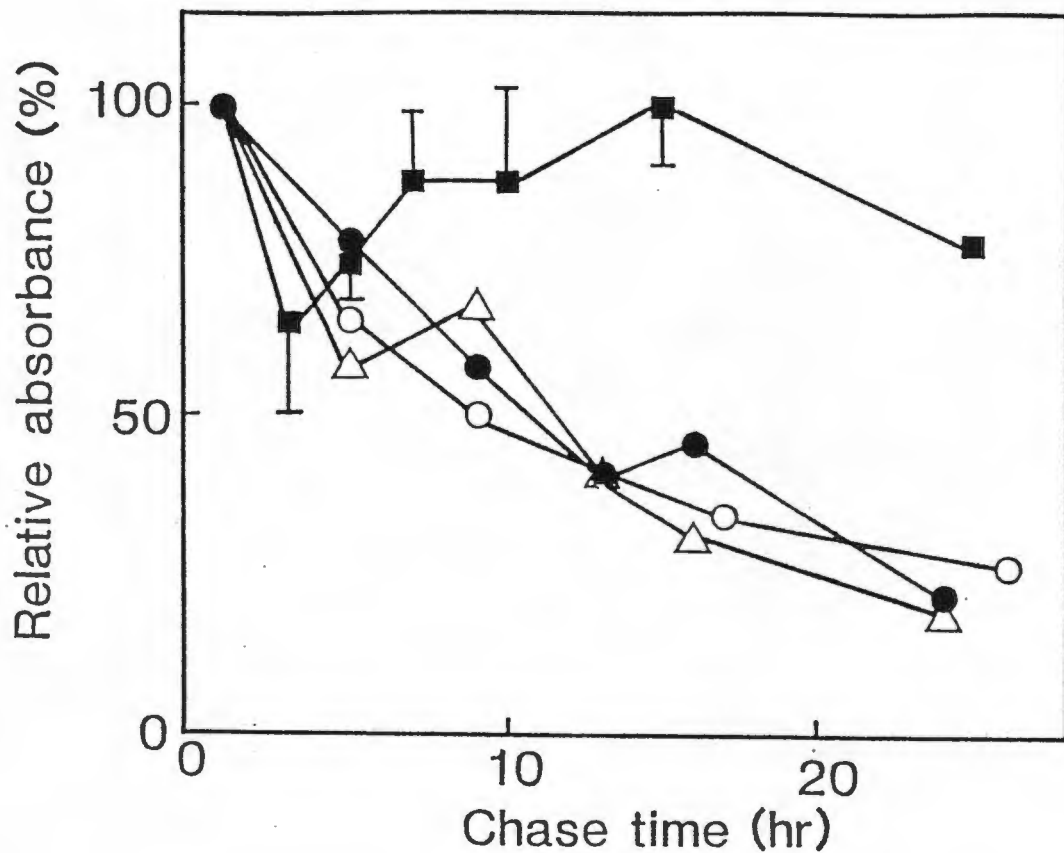
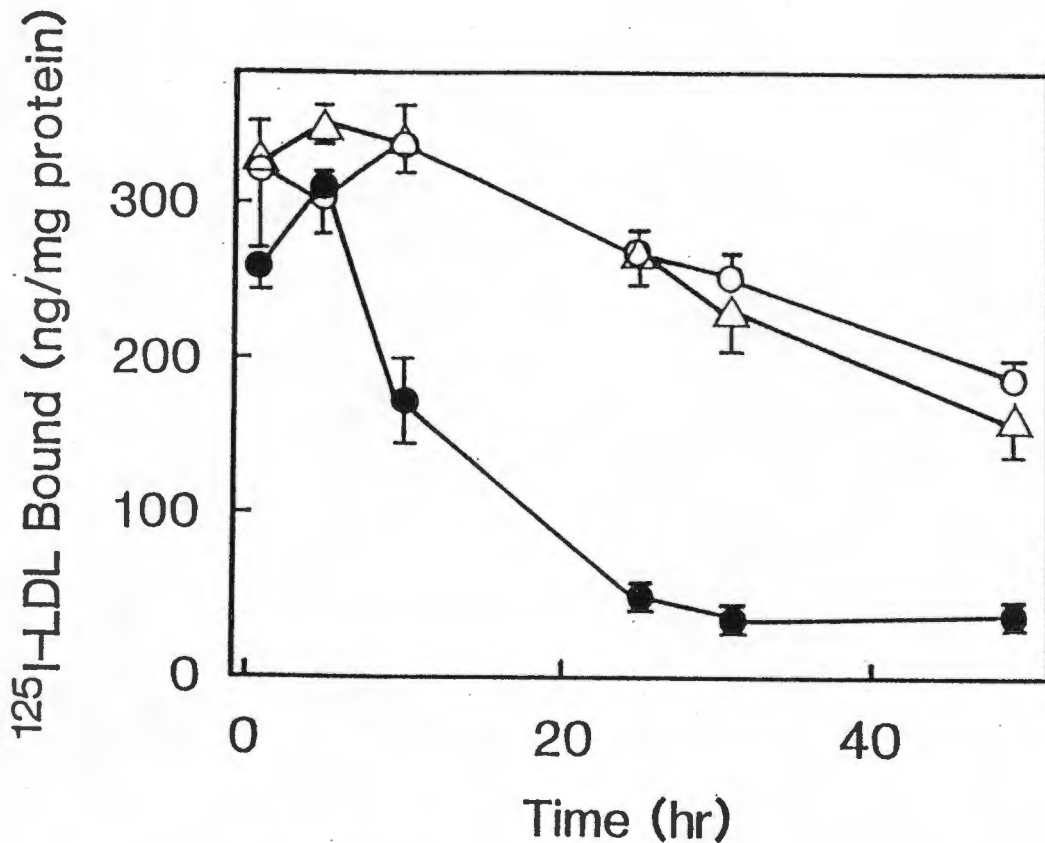


Fig. 4.6: The degradation rate of  $^{35}\text{S}$ -labeled LDL receptors from normal fibroblasts in the presence of various agents. The cell monolayers were upregulated for 16 hours and pulse-labeled with  $50 \mu\text{Ci/ml}$  of  $[^{35}\text{S}]$ -methionine, and then chased for the indicated times in complete DMEM/LPDS medium supplemented with : no further addition (●),  $50 \mu\text{g/ml}$  LDL (○),  $1 \mu\text{g/ml}$  25-hydroxycholesterol ( $\Delta$ ) or  $100 \mu\text{M}$  cycloheximide (■). The solubilized cell extracts were then analysed as described in "Experimental Procedures". Values plotted represent the percentage absorbance, relative to the first (1 hr) chase point, at each time, and are the mean  $\pm$  S.D. of results obtained from triplicate precipitations in the case of cycloheximide, or are the means obtained from duplicate incubations (●,○,Δ). Duplicates never differed by more than 15% from the means. Each curve represents a single experiment, which typifies results obtained from at least 2 other such experiments.

#### 4.3.6 Surface LDL receptors, as assayed after the addition of various agents.

The expression of LDL receptors on HSF, as assessed by surface binding of  $^{125}\text{I}$ -LDL, was next studied (Fig. 4.7). The LDL receptors in the monolayers were fully upregulated prior to commencing the experiment (48 hours), and the cells were then incubated for various times at  $37^\circ\text{C}$  in medium supplemented with the indicated agent(s). At each time, high-affinity cell surface receptor activity was assayed. Incubation in the presence of 25-hydroxycholesterol resulted in a loss of surface receptors after an initial lag period of about 5 hr. The kinetics of LDL receptor loss were apparently first order thereafter, with a  $t_{1/2}$  of 13 hr, similar to the  $t_{1/2}$  value obtained using the [ $^{35}\text{S}$ ]-pulse-chase protocol. In line with the results reported in Fig. 4.6 and Fig. 4.5, cycloheximide markedly reduced surface LDL receptor loss, irrespective of whether 25-hydroxycholesterol was present or not.

From the above, it is clear that 25-hydroxycholesterol affected the synthesis of LDL receptors only, and had no apparent effect on LDL receptor degradation. Since 25-hydroxycholesterol enhanced the degradation of HMG CoA reductase (40), it is conceivable that this agent might affect cellular degradation systems on a wider scale; LDL receptors are, perhaps, merely an exception to this general rule. To investigate this, the effect of  $0.6\ \mu\text{g}/\text{ml}$  25-hydroxycholesterol on the endogenous long- and short-lived protein turnover rates in normal lymphoblastoid cells was

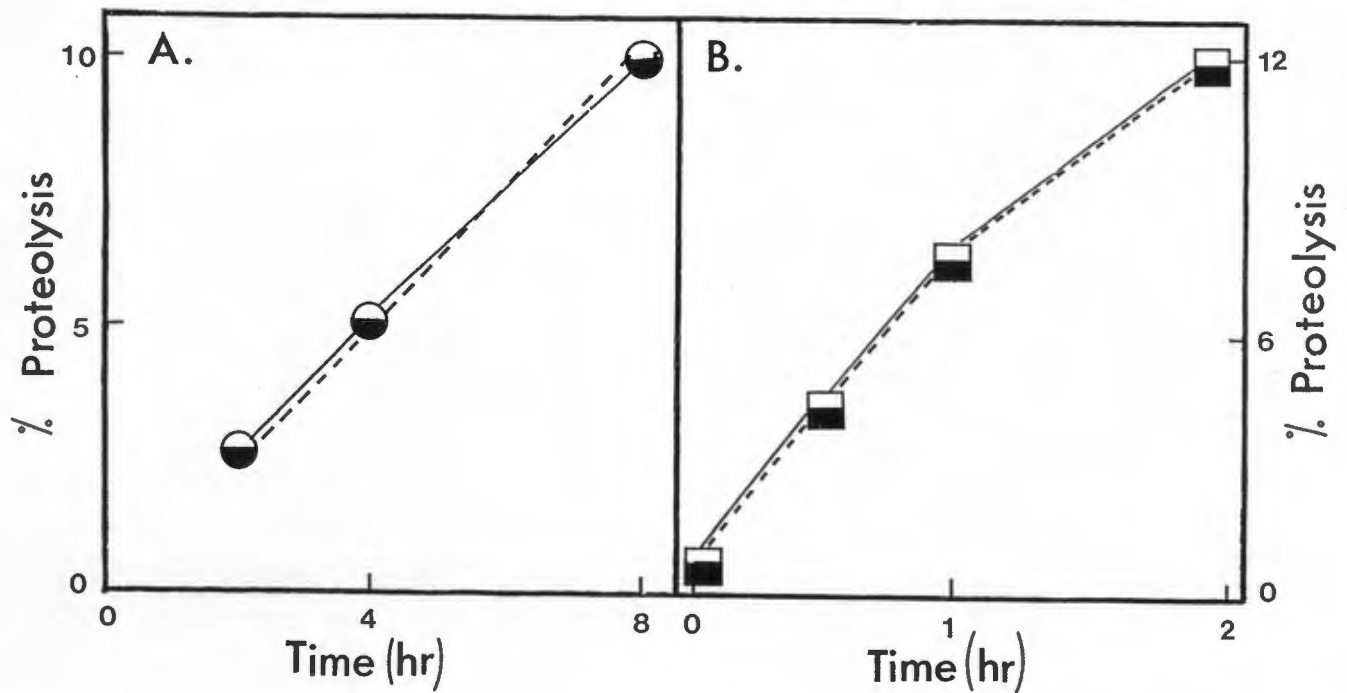


**Fig. 4.7: Surface LDL receptors in fibroblast monolayers.** On day 7 of cell growth, after incubation for 48 hr in medium containing lipoprotein-deficient serum, each monolayer received a fresh 2 ml of this medium containing the following agent(s): 1  $\mu$ g/ml 25-hydroxycholesterol (●), 100  $\mu$ M cycloheximide (○) or 1  $\mu$ g/ml 25-hydroxycholesterol + 100  $\mu$ M cycloheximide ( $\Delta$ ). Monolayers were maintained at 37°C for the indicated times, after which <sup>125</sup>I-LDL binding was performed. All incubations containing <sup>125</sup>I-LDL only were performed in triplicate, while those containing an excess of LDL were done in duplicate. The results represent a single experiment, and are typical of several identical experiments. Non-specific binding was always < 10% of the total binding in the fully upregulated state.

examined. Intracellular proteolysis was measured by the release of labeled amino acids into the cellular incubation media, as described in section 4.2.5. The long-lived proteins were degraded at a rate of 1.3% per hour (Fig. 4.8A), while 8% of the short-lived proteins were degraded per hour (Fig. 4.8B). The addition of 25-hydroxycholesterol altered neither of these rates. Parallel binding incubations performed on cells which had been maintained in upregulation medium containing 25-hydroxycholesterol (using the same stock solution as for the previous rates), showed a significant reduction of surface LDL receptors as obtained previously (Fig. 4.5). This indicates that the lack of effect of the 25-hydroxycholesterol on the proteolysis rates was not due to an inactive stock solution. Similar basal degradation rates have been reported by other investigators in this laboratory using cultured smooth muscle cells: Bates *et al* (17) obtained a degradation rate for long-lived proteins of 1.5% per hour in smooth muscle cells, and one of 6.5% per hour for short-lived proteins.

#### 4.3.7 The half-life of LDL receptors in internalization-defective mutant cells.

The next question addressed was whether the turnover rate of the LDL receptor protein was in any way related to the fact that the receptor normally recycles in and out of cells. The GM 2408A fibroblast line (corresponding to patient J.D.) bound <sup>125</sup>I-LDL normally, but, as expected, showed greatly reduced internalization and degradation of the ligand (Table



**Fig. 4.8:** Time course of general protein degradation in normal lymphoblastoid cells. (A), long-lived proteins (circles); (B), short-lived proteins (squares).

Cultured lymphoblastoid cells were incubated for 43 hours with medium containing 1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-phenylalanine to preferentially label the long-lived proteins (A), or for 1 hour with medium containing 4  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-phenylalanine to label the short-lived proteins (B). The cells were then washed, and proteolysis was measured in the absence (open symbols, dashed lines) or presence (closed symbols, solid lines) of 0.6  $\mu\text{g/ml}$  25-hydroxycholesterol at the indicated times, as described under "Experimental Procedures" (section 4.2.5). All incubations were performed in duplicate, and duplicates never differed by more than 5% from the means.

**Table 4.1:  $^{125}\text{I}$ -LDL metabolism with LDL receptor internalization defective fibroblasts (GM 2408A) and normal fibroblasts (GM 3348A).**

On day 7 of cell growth, after incubation for 48 hr in DMEM/LPDS medium, each monolayer received 2 ml fresh medium containing 10  $\mu\text{g/ml}$   $^{125}\text{I}$ -LDL in the absence or presence of 200  $\mu\text{g/ml}$  unlabeled LDL. After incubation for 4 hr at 37°C, the high-affinity values for surface-bound, intracellular and degraded  $^{125}\text{I}$ -LDL were measured and calculated. Incubations were performed in duplicate. Duplicate values never differed by more than 5% from the means.

Handling Parameter	$^{125}\text{I}$ -LDL (ng/mg protein)	
	GM 3348A	GM 2408A
Surface bound	124	141
Intracellular	765	128
Degraded	2969	111

4.1). This internalization-defective phenotype is known to be caused by the substitution of cysteine for tyrosine at residue 807 in the LDL receptor (51), which may alter the conformation of the cytoplasmic tail, resulting in the inability of the receptors to cluster in coated pits on the cell surface (5). The mutant receptors remain randomly scattered over the entire plasma membrane (200), do not recycle, and thus provide a model system for non-recycling LDL receptors. The turnover rate of  $^{35}\text{S}$ -labeled LDL receptors was measured in cells from this line, using the standard pulse-chase protocol followed by immunoprecipitation with preformed immune complexes containing IgG-C7: this gave a first order decay curve with a  $t_{1/2} = 10.5$  hr (Fig 4.9). This value was not significantly different ( $P > 0.05$ ) from that determined for actively recycling LDL receptors in normal fibroblasts, in the presence of LDL. These results indicate that the normal degradation pathway for the LDL receptor is not dependent on receptor clustering, internalization or recycling.

#### 4.3.8 The nature of the proteolytic system(s) involved in the degradation of LDL receptors.

In an attempt to identify the nature of the rate-limiting proteolytic entities which might initiate LDL receptor degradation, the effects on the receptor population of various agents known to affect mainly lysosomal proteolysis, were examined. First, the behaviour of cell-surface receptor activities was measured. The data obtained,

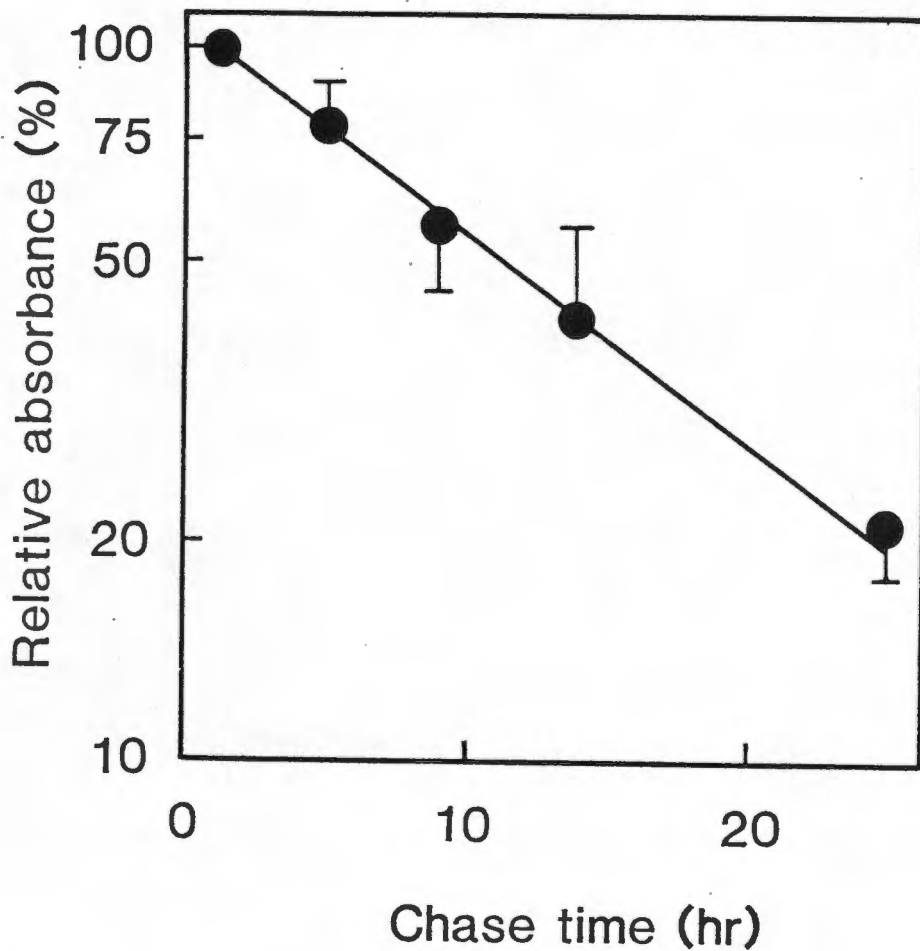


Fig. 4.9: Turnover of  $^{35}\text{S}$ -labeled LDL receptor from GM 2408A.

Monolayers of GM 2408A were incubated for 16 hr with DMEM/LPDS, then pulse-labeled for 8 hr in EMEM/LPDS containing 40  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine. The chase-incubations, solubilizations and immunoprecipitations with IgG-C7 were as described in the legend to Fig. 3.11. A semilog plot, of % relative absorbance (absorbance of LDL receptor at each time, relative to that at one hour) versus chase time, was constructed. Values plotted represent mean  $\pm$  S.D. of triplicate immunoprecipitations at each point. The results shown were from a single experiment, representative of 2 such experiments, performed on separate days.

summarized in Table 4.2, show that the addition of 150  $\mu\text{g/ml}$  leupeptin to normal fibroblasts for either 1 or 5 hr at 37°C did not change the number of surface LDL receptors relative to untreated controls. The addition of 70  $\mu\text{M}$  chloroquine produced a decrease of about 30% relative to controls at both times, while 10 mM ammonium chloride decreased  $^{125}\text{I}$ -LDL binding by 60% after 1 hr and by 80% after 5 hr. Treatment with 25  $\mu\text{M}$  monensin produced the most marked effects, decreasing the surface LDL receptors by 82% at 1 hr and by 96% after 5 hr at 37°C. In all cases, 40  $\mu\text{g/ml}$  LDL was present in the 37°C incubation media, to facilitate the internalization of the receptor, an effect which has been well documented in the case of monensin (15). The 37°C incubation times of 1 and 5 hr were chosen since little downregulation of the LDL receptors has been observed to occur during periods as short as these (Note: the lag phase for downregulation in the presence of LDL is 5 hr). Because leupeptin caused no apparent decrease in the number of surface LDL receptors, it was considered necessary to demonstrate that this agent in fact inhibited lysosomal degradation under the conditions used.  $^{125}\text{I}$ -LDL was accordingly incubated with normal fibroblast monolayers in the absence or presence of leupeptin for 4 hr at 37°C (Table 4.3). While surface  $^{125}\text{I}$ -LDL binding remained unaltered, the intracellular  $^{125}\text{I}$ -LDL content increased by 76% and the degradation rate of radioactive ligand was inhibited by 54%.

The effects of these inhibitors were also analysed in [ $^{35}\text{S}$ ]-methionine pulse-chase experiments in order to compare the absolute turnover rates of the receptors with the binding

**Table 4.2: The effect(s) of various agents on the surface LDL receptors in normal fibroblasts.**

On day 7 of cell growth, after 48 hr in DMEM/LPDS, each monolayer received 2 ml/dish fresh DMEM/LPDS + 40  $\mu\text{g/ml}$  LDL, supplemented with the additions listed in the Table. After incubating the monolayers for either 1 or 5 hr at 37°C,  $^{125}\text{I}$ -LDL (10  $\mu\text{g/ml}$ ) was bound to the cells at 4°C for 1.5 hr in the absence or presence of unlabeled LDL (200  $\mu\text{g/ml}$ ), and high-affinity  $^{125}\text{I}$ -LDL binding values were calculated. The data were obtained from a single experiment, with duplicate dishes. Duplicates never differed by more than 5% from the means. Non-specific values were less than 10% of the total in the absence of additions, and these values remained approximately constant after each of the indicated additions.

Addition	$^{125}\text{I}$ -LDL bound (ng/mg protein) after adding agent(s) for:	
	1 hr	5hr
no addition	361	331
leupeptin (150 $\mu\text{g/ml}$ )	334	350
chloroquine (70 $\mu\text{M}$ )	262	237
ammonium chloride (10 mM)	145	66
monensin (25 $\mu\text{M}$ )	66	12

**Table 4.3: The effect(s) of leupeptin on  $^{125}\text{I}$ -LDL metabolism in normal fibroblasts.**

On day 7 of cell growth, after 48 hr in DMEM/LPDS, each monolayer received 2 ml/dish fresh DMEM/LPDS containing 10  $\mu\text{g/ml}$   $^{125}\text{I}$ -LDL with or without 200  $\mu\text{g/ml}$  LDL, in the absence or presence of 150  $\mu\text{g/ml}$  leupeptin. The monolayers were incubated for 4 hr at 37°C, and high-affinity surface bound, intracellular and degraded  $^{125}\text{I}$ -LDL were determined. All incubations containing  $^{125}\text{I}$ -LDL only ( $\pm$  leupeptin) were performed in triplicate, while those done in the presence of excess unlabeled LDL were done in duplicate. Values are means  $\pm$  S.D.. This experiment was performed concurrently with that reported in Fig. 4.10, using the same leupeptin stock solution.

Agent present during incubation	$^{125}\text{I}$ -LDL (ng/mg cell protein)		
	Bound	Intracellular	Degraded
-	382 $\pm$ 8	2449 $\pm$ 119	1881 $\pm$ 118
leupeptin (150 $\mu\text{g/ml}$ )	413 $\pm$ 35	4324 $\pm$ 479	867 $\pm$ 58

data reported above. The agents were added, at the same concentrations as tested previously, to the chase media of fibroblasts. Chloroquine, ammonium chloride and monensin all produced the same effect - an extremely rapid and marked decrease in receptor protein (as detected by immunoprecipitation with IgG-C7), such that in each case less than 20% of the labeled receptors remained after 8 hr (Fig. 4.10A). In contrast, the presence of leupeptin in the chase media was associated with a decay curve which did not differ significantly ( $P > 0.05$ ) from that obtained in the absence of the inhibitor (Fig. 4.10A). The simultaneous addition of leupeptin and monensin to the chase media produced a significant ( $P < 0.0001$ ) decrease in the rate of LDL receptor loss, relative to that obtained in the presence of monensin only (Fig. 4.10B). The presence of all these agents in the chase media did not result in a marked alteration in the degradation rate of transferrin receptors. After a 15 hr chase in the absence or presence of monensin,  $\text{NH}_4\text{Cl}$ , chloroquine or leupeptin, the relative amounts of transferrin receptors compared to those present at the start of the chase-incubations were 90%, 100%, 100%, 85% and 83%, respectively. Because of the latter finding, the amount of  $^{35}\text{S}$ -labeled LDL receptors was also expressed relative to the radioactivity in the transferrin receptor bands (Fig. 4.10B), to control for variations encountered in the isolation process.

Lysosomal proteolysis of externally added ligands is totally inhibited at  $18^\circ\text{C}$  (10, 67, 152). This is probably due to the inability of lysosomal membranes to fuse with those of

Relative absorbance  
(LDL Receptor/transferrin receptor)

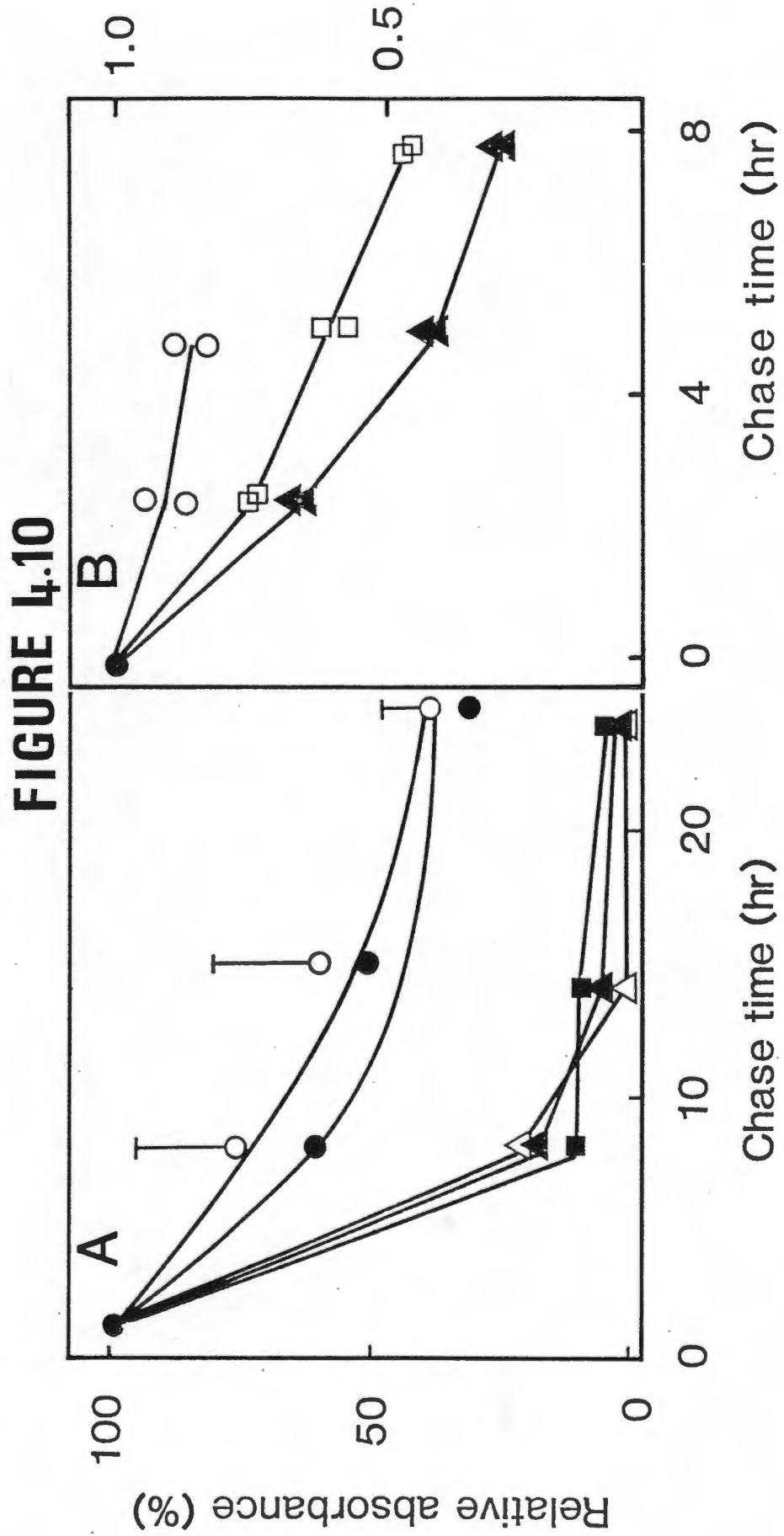


Fig. 4.10: The degradation of LDL receptors from normal fibroblasts in the absence or presence of various agents. Monolayers were pulse-labeled for 7 hr in EMEM/LPDS containing 50  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine, then chase-incubated for the indicated times in complete DMEM/LPDS medium + 40  $\mu\text{g/ml}$  LDL containing the following agents: no further addition ( $\bullet$ ); 150  $\mu\text{g/ml}$  leupeptin ( $\circ$ ); 70  $\mu\text{M}$  chloroquine ( $\Delta$ ); 10 mM ammonium chloride ( $\blacksquare$ ); 25  $\mu\text{M}$  monensin ( $\blacktriangle$ ) or 25  $\mu\text{M}$  monensin + 150  $\mu\text{g/ml}$  leupeptin ( $\square$ ). Immunoprecipitations were performed with preformed immune complex containing IgG-C7 and B3/25, and the results were analysed as described in "Experimental Procedures". Values plotted represent the amounts of LDL receptor present at each time, expressed as a % of that present at the first point. In Exp A the values are either the means  $\pm$  S.D. of results obtained from triplicate incubations ( $\circ$ ) or are the means obtained from duplicate incubations ( $\bullet$ ,  $\blacksquare$ ,  $\blacktriangle$ ,  $\Delta$ ). Duplicates never differed by more than 20% from the means. Each curve represents the results obtained from a single experiment, which in some cases ( $\circ$ ), was typical of results obtained in 3 similar experiments, performed on separate days. In Exp B the values plotted were calculated by expressing the amount of LDL receptor present at each time relative to the amount of transferrin receptor present at the same time. This compensated for differential losses during the isolation procedure of the receptors. Duplicate dishes (each value plotted) from a single experiment were analysed at each point.

endocytic vesicles at this temperature. The effect of an 18°C chase-incubation on the turnover rate of <sup>35</sup>S-labeled LDL receptors was examined (Fig. 4.11). Degradation rates were still clearly detectable but were slower compared to the rates at 37°C: it took 50-70 hours for 50% of the receptors to be degraded, compared with the 12 hours taken to achieve a similar reduction at 37°C. That the decrease in labeled LDL receptors detected after the chase-incubations at 18°C in fact represented the turnover of this protein (and not loss of cell viability or cell death), was confirmed by (i) visual inspection of the monolayers under the phase-contrast microscope and (ii) comparing the turnover rate of LDL receptors with that of the transferrin receptors, at 18°C. As is illustrated in Fig. 4.11, the rate of degradation of the transferrin receptors was also markedly decreased, which is consistent with a simple temperature effect on the protein turnover processes in viable cells.

#### 4.3.9 The effect of neuraminidase treatment on the LDL receptor half-life.

LDL receptors are enriched in sialic acid residues on their extracellular aspect (46). To determine whether these residues are important in determining the rates of receptor degradation, fibroblast monolayers were pulse-labeled with [<sup>35</sup>S]-methionine for 7.5 hr. The intact cells were then incubated for 30 min at 37°C in the absence or presence of neuraminidase, after which the monolayers were chase-

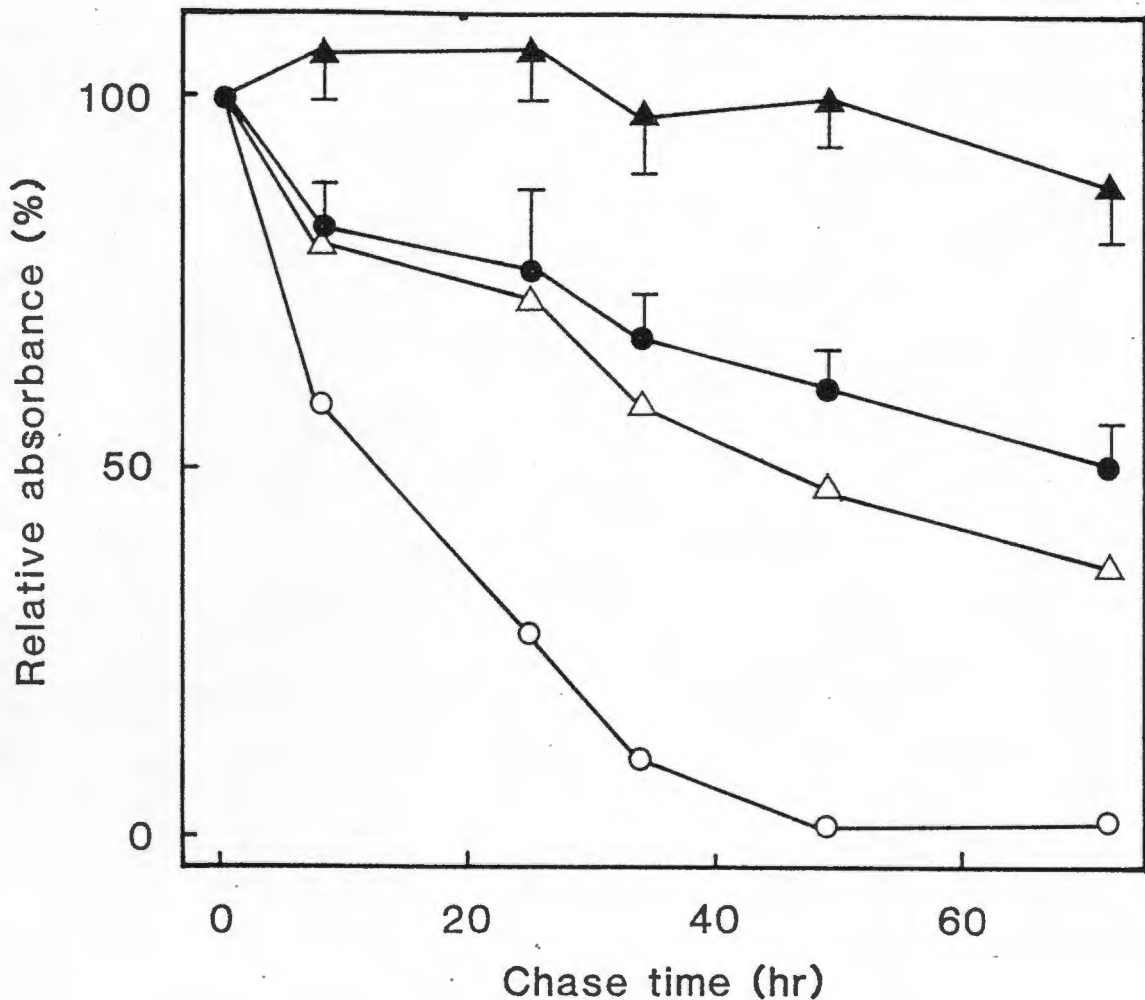


Fig. 4.11: Degradation of  $^{35}\text{S}$ -labeled LDL receptors (●,○) and  $^{35}\text{S}$ -labeled transferrin receptors (▲,△) from normal fibroblasts at various temperatures.

The cell monolayers were pulse-labeled for 8 hr in EMEM/LPDS medium containing  $50 \mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine, then chase-incubated for the indicated times in complete DMEM/LPDS medium at either  $18^\circ\text{C}$  (closed symbols) or  $37^\circ\text{C}$  (open symbols). After detergent solubilization, the cell extracts were incubated with preformed immune complexes containing IgG-C7 and B3/25, to precipitate the LDL (●,○) and transferrin receptors (▲,△), respectively. Precipitations after chase-incubating at  $18^\circ\text{C}$  were done in triplicate, while single precipitations were performed at each time after the  $37^\circ\text{C}$  chase. All 4 curves were obtained from the same single experiment.

incubated (Fig. 4.12). Neuraminidase treatment, which removes the terminal sialic acid residues from both the N- and O-linked oligosaccharide chains of mature receptors, produced a decrease in the apparent molecular weight of the receptors of about 10-12 kd. The half-life of the desialylated mature LDL receptors (13 hr) did not differ significantly ( $P > 0.05$ ) from that of sialylated receptors (Fig. 4.12B), showing that the presence or absence of the sialic acid residues played no role in determining the rate of degradation of the receptor proteins. It is noteworthy that receptors did not become resialylated for the duration of the 24 hr subsequent to the neuraminidase treatment. In addition, it is likely that sialic acid residues were removed from different types of cell surface glycoproteins, since the neuraminidase treatment was performed on whole cells. From this, it can be inferred that the other cell surface glycoproteins do not need terminal sialic acid residues if they are somehow involved in LDL receptor turnover.

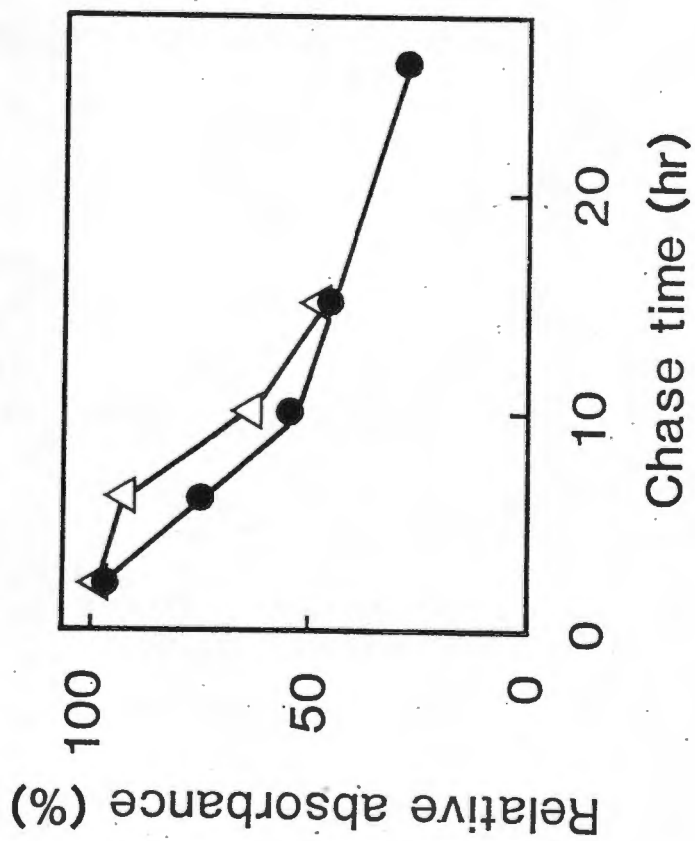
#### 4.4 Discussion.

Using the pulse-chase protocol (as described in Section Three) on cultured human skin fibroblasts, a half-life of  $11.7 \pm 2.2$  hr for the LDL receptor was obtained (section 3.9). In this study, the turnover of LDL receptors (and the implications for LDL receptor up- and downregulation), were analysed, and the possible degradation mechanisms were investigated.

FIGURE 4.12

Pre-chase treatment	+ neuraminidase						-neuraminidase				
	2	6	10	15	24		2	6	10	15	
Chase time (hr)											
Mature LDL-R ( $\pm$ sialic acid residues)											

A.



B.

Fig 4.12: Degradation of  $^{35}\text{S}$ -labeled LDL receptors from fibroblasts either with (●) or without (Δ) neuraminidase treatment.

Monolayers were pulse-labeled for 7.5 hr in EMEM/LPDS medium containing 50  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine. The cells were washed, then 1.3 ml of buffer containing 50 mM sodium citrate, pH 6, 100 mM NaCl and 2 mM  $\text{CaCl}_2$  was added to each dish. In some cases, 50  $\mu\text{l}$  neuraminidase (containing 0.05 enzyme units) was added to the appropriate dishes (●) while in other cases it was omitted (Δ). All monolayers were incubated at 37°C for 30 minutes, then washed twice with PBS before adding the chase medium (complete DMEM/LPDS containing 200  $\mu\text{M}$  methionine). At the indicated times, the chases were terminated and the solubilized cell extracts were incubated with preformed immune complexes containing IgG-C7. (A) Fluorograms showing the 160 kd sialylated mature LDL receptor (- neuraminidase treatment) and the 150 kd receptor protein obtained (+ neuraminidase treatment) (B) Linear plot, showing the absorbance of the extracted radioactive LDL receptor band at each time, expressed as a percentage of the absorbance obtained at the first time point (2 hr). Immunoprecipitations at each point were performed on duplicate dishes (+ neuraminidase) or on single dishes (- neuraminidase). Duplicates never differed by more than 10% from the means.

It was important to consider whether the half-life of the LDL receptor was affected by the considerable difference in LDL receptor concentrations in the up- and downregulating states, and whether the mechanism(s) of the LDL receptor regulation involved changes in the relative turnover rates. This was tested directly by performing the experiment depicted in Fig. 4.1. The results showed unequivocally that the half-lives of LDL receptors remained the same over the range of cellular LDL receptor protein content that was tested.

#### LDL receptor up- and down regulation.

Direct proof that LDL receptor regulation is a function of changes solely in the receptor synthetic rate, was obtained by generating theoretical curves using first order kinetics with a half-life for the LDL receptor of  $11.7 \pm 2.2$  hr, and then comparing these curves with the experimental data (Fig. 4.2A). The following important considerations underly the use of this model: (i) the LDL receptor half-life is independent of the concentration of the receptor. This was clearly shown to be true over the range of LDL receptor concentration which was examined (Fig. 4.1). This means that the degradation rate of the receptor protein depends linearly on the LDL receptor concentration, assuming the rate-determining step in the degradation process to be either highly undersaturated or non-saturable; (ii) after initiating the upregulation process, the time course of reaching the new steady state depends solely on the half-life of the receptor, while the actual level depends both on

the receptor half-life and on the magnitude of the increased synthetic rate of the receptor protein. The fact that the experimental curves for up- and downregulation kinetics were mirror images indicates conclusively that up- and downregulation of the LDL receptor is due only to changes in the rates of receptor synthesis. In contrast, Knight et al (128) have recently reported that incubation of fibroblasts in LPDS produced an initial increase in the rate of LDL receptor synthesis, followed by a decrease of over 60% to a new constant value; this decrease was not entirely inhibited by compactin. Such a decrease in receptor synthesis during the period of upregulation of the receptors was not obtained in the studies described here (Fig. 4.2B): no explanation for this difference can yet be offered.

It is very interesting that insulin-induced downregulation of the insulin receptor is caused solely by an accelerated receptor degradation rate, with synthetic rates remaining unaltered (120, 195), whereas the decrease in HMG CoA reductase in the presence of 25-hydroxycholesterol is due both to a decreased synthetic and an increased degradation rate in respect of the protein (78, 235).

#### **A putative degradation intermediate.**

A putative intermediate was identified in the LDL receptor degradation pathway of apparent molecular weight approximately 120 kd (Fig. 4.3), using a polyclonal antibody directed against the LDL receptor. This fragment was not recognized by the monoclonal antibody, IgG-C7, which is specific for the NH<sub>2</sub>-terminal ligand-binding domain of LDL

receptors. This suggests that the large degradation intermediate represents a proteolytically processed form of the LDL receptor which has lost part of the amino-terminal domain. It is also possible, but less likely, that some as yet unidentified factor in the initial stages of the degradative pathway may have produced a conformational change in the intact 160 kd receptor, prior to proteolytic cleavage, resulting in a loss of IgG-C7 recognition and a concomitant apparent decrease in molecular weight of the protein as observed on SDS-PAGE. An analogous intermediate has been previously observed in experiments where a polyclonal antibody, directed against a synthetic peptide corresponding to the COOH-terminal 14 amino acids of the LDL receptor, was used for immunoprecipitation (140).

#### LDL receptor turnover in two other cell types.

The turnover rate of LDL receptors on the well-established human hepatoma cell line, Hep G2, was measured (Fig. 4.4). This cell type was chosen since it is widely regarded, and generally used, as a good model system to study the catabolism of LDL by human hepatocytes in vitro (96), and because the liver is known to play an important role in LDL uptake from the circulation. The half-life of the LDL receptor in the Hep G2 cells was not significantly different from that obtained in HSF, indicating that the half-life of 11.7 hr in the latter cell line is probably not a tissue-specific feature.

The half-life of the LDL receptor in cultured human lymphoblastoid cells was about 4 hours, as assayed by

cell-associated  $^{125}\text{I}$ -LDL after preincubating the cells for various times in either LDL only, or in LDL + 25-hydroxycholesterol (Fig. 4.5). This value was significantly shorter than that obtained for these receptors in HSF or Hep G2 cells. When preincubations were performed in the presence of LDL supplemented with cycloheximide, the degradation rate of the receptor was significantly decreased, as was the case, although to a lesser extent, when LDL, 25-hydroxycholesterol and cycloheximide were added simultaneously. This general trend was similar to that noted with the fibroblast monolayers (section 4.3.6).

#### Independent routing of receptor and ligand towards degradation.

The pulse-chase experiments which were performed in the presence of LDL (Fig. 4.6) have provided evidence for independent routing of receptor and ligand towards degradation, since the addition of LDL to the chase medium yielded a receptor half-life which did not differ significantly from that obtained in the absence of the ligand. This is in agreement with the widely accepted role of the LDL receptor in receptor-mediated endocytosis (77, 88). Similar pathways are traversed by many other receptors and their ligands, such as the asialoglycoprotein receptor (28, 256), the half-life of which has also been shown to remain unaffected *in vivo* in the presence of its ligand (233). In contrast, the degradation of several other receptors has been shown to be drastically altered in the presence of their ligands e.g. when EGF is added to cells, the receptors are internalized and degraded with the ligand,

resulting in a marked decrease in the half-life of the receptors (55, 65).

#### The effect of cycloheximide.

The initial transient decrease in the number of LDL receptors which was obtained in the presence of cycloheximide (Fig. 4.6) suggests that a rapidly turning over protein is necessary for a rate-limiting step in the degradation of the LDL receptor. After blocking the synthesis and depleting the intracellular pool of this putative short-lived protein by adding cycloheximide, the dramatically decreased rate of receptor loss would account for the apparently constant content of labeled receptors at chase times  $> 5$  hr. An analogous effect on the insulin receptor (i.e. initial rapid degradation, followed by a very reduced rate of receptor turnover) has been documented by Reed et al (190) using either cycloheximide or puromycin in 3T3-L1 adipocytes. That puromycin also decreased degradation indicates that the cycloheximide effect is due to an inhibition of protein synthesis itself, and is not due to a specific effect of a particular inhibitor on receptor synthesis. Additional support for this mode of cycloheximide action is provided by Stimac et al (227), who have shown that the increased stability of histone mRNA in exponentially growing S49 cells in the presence of cycloheximide results from loss of an activity which regulates mRNA turnover in the cell, and is not due to an alteration in the mRNA structure. Kusari and Sen (136) have shown that the rapid turnover of interferon-inducible mRNA in HeLa cells is blocked by either cycloheximide or

actinomycin D, an effect which they concluded was due to the need for protein synthesis to bring about catabolism of the mRNA.

#### The effect of 25-hydroxycholesterol.

In the presence of 25-hydroxycholesterol, an exponential loss of high-affinity cell surface receptors was detected by  $^{125}\text{I}$ -LDL binding, after an initial 5 hr lag period (Fig. 4.7). The decay curve was identical to that obtained after incubating cells for various times in medium containing LDL (Fig. 4.2A). Both curves displayed first order kinetics, and yielded half-lives of about 13 hr, similar to that obtained using the  $^{35}\text{S}$ -pulse-chase procedure. It is likely that the sterols strongly repressed the synthesis of the LDL receptor [by repressing the gene for the LDL receptor (77, 199)], during which time normal receptor turnover brought about a reduction in the number of surface receptors. A marked reduction in surface LDL receptor loss was also obtained in the presence of cycloheximide, in both the absence or presence of 25-hydroxycholesterol, which is consistent with the results depicted in Fig. 4.6 and discussed above.

#### Receptor Recycling.

The pathways and processes involved in the degradation of the LDL receptor are unknown. It is generally accepted that some or most endocytosed LDL receptors recycle to the cell surface via a pre-lysosomal route ("short loop"). Brown et al (30) have speculated that a small proportion of the receptor population could be partitioned into the lysosome-

targeted route, leading either to degradation of receptors, or to passage through a separate salvage pathway involving the trans-golgi reticulum ("long loop"), before returning to the cell surface. Strong evidence is now provided, from pulse-chase experiments on the LDL receptor internalization-defective fibroblast cell line (GM 2408A, from patient J.D.) (Fig. 4.9), that participation of the receptor in the receptor-mediated endocytotic pathway is not an essential prerequisite for degradation of the receptor at the rates observed in normal cells.

#### Possible degradation mechanism(s).

A useful way of distinguishing between lysosomal and extralysosomal protein degradation pathways in intact cells depends on the use of proteinase inhibitors, lysosomotropic agents or ionophores. To this end, chase-incubations, in the absence or presence of the following agents, were performed (Tables 4.2 & 4.3 and Fig 4.10): leupeptin, an inhibitor of lysosomal cathepsins B, H and L (168) and of calpains (245); chloroquine or ammonium chloride, weak bases which increase the pH of acidic intracellular compartments (54); and monensin, a carboxylic ionophore, which among other effects, is known to increase intravesicular pH (237). All the agents except leupeptin caused a significant and rapid redistribution of the surface LDL receptors to intracellular locations; corresponding pulse-chase experiments showed an extremely rapid, marked decrease in labeled LDL receptors. Leupeptin produced neither of these effects. It is likely that the former group of agents affected cells in such a way that the

concentration of lysosomal enzymes in certain cellular vesicles was increased, or indeed the number of lysosomes - effects which have been noted by others (226). On examination under the phase-contrast microscope, fibroblasts incubated in the presence of monensin, ammonium chloride or chloroquine were observed to assume a distinctly "bubbly" morphology, possibly due to "proliferation" and expansion of various kinds of vesicles. It is unlikely that these findings prove that receptors are mostly degraded in lysosomes. The following possible explanation is proposed for the observed events: since these agents caused an apparent receptor redistribution to the intracellular vacuolar compartment (Table 4.2), and because there may have been an increased concentration of lysosomal enzymes in this system, the relocated receptors may have been rapidly degraded by enzymes able to operate at the pH levels in the particular locations.

An alternate plausible explanation (which may, or may not, occur in addition to the above proposed degradation mechanism) for the observed rapid loss of LDL receptors detected after incubation in the presence of chloroquine, ammonium chloride or monensin, could be that the receptors merely undergo a partial denaturation (or even just a subtle conformational change) that results in the loss of recognition for IgG-C7. This could account for the rapid loss of the 160 kd LDL receptor bands, as is illustrated in Fig. 4.10A. When leupeptin and monensin were added simultaneously to the chase media, the rate of LDL receptor loss decreased significantly but only partially (relative to

that obtained with monensin only) (Fig. 4.10B). The leupeptin-inhibitable receptor losses caused by monensin may represent actual receptor degradation, whereas the monensin-induced loss of receptors which was not inhibitable by leupeptin could be due to a partial denaturation of receptors, accompanied by a concomitant loss of IgG-C7 recognition. The experimental data obtained to date cannot distinguish between the possibilities of degradation and/or partial denaturation resulting in the observed LDL receptor loss in the presence of the above-mentioned agents.

In a recent study by Davis et al (50), deletion of the domain of the receptor that contains "growth factor repeats" was associated with inability of the receptor to dissociate from LDL at acid pH; as a result, the receptor was no longer recycled efficiently, and was rapidly degraded after ligand binding.

Leupeptin, which neither altered the surface distribution of the LDL receptor, nor produced any visible proliferation of large vesicles, had no effect on receptor turnover. It appears that the proteases which are inhibited by leupeptin (amongst which are the non-lysosomal calpains) do not play a rate-limiting role in the degradation of the LDL receptor, excepting when cells are severely disturbed by drugs which prevent normal vesicular function.

Aulinskas et al (10) have demonstrated that at temperatures between 10 and 20°C, LDL receptors on HSF retain the ability to bind and internalize LDL, and to recycle to the cell

surface, but degradation of the ligand, (a lysosomal event), is completely inhibited. This lack of degradation was probably due to inhibition of fusion between lysosomes and endocytosed vesicles at the reduced temperatures (67, 152). LDL receptors were degraded at this temperature at a rate commensurate with the approximated four-fold slowing down of chemical processes when stepped down from 37°C to 18°C. This observation suggests that inhibition at least of LDL receptor degradation is non-lysosomal; this contrasts with the notion that receptor degradation is a lysosomal event, associated somehow with ligand targeting to lysosomes (for review, see (247)). The possibility that "downstream" steps in the pathway for degradation of LDL receptors occur within the lysosomes, cannot be excluded at this stage. The failure of leupeptin to alter the turnover rate of the receptor is not conclusive evidence against this notion, given that the "downstream" steps may not become rate-limiting until they are inhibited to a very severe degree. The possible non-lysosomal mechanisms of receptor degradation are discussed more fully in Section Five.

#### **Desialylation of the mature LDL receptor.**

Many membrane proteins contain oligosaccharide chains which are covalently attached to either asparagine (N-linked) or to serine and threonine (O-linked). The precise function of the oligosaccharide moieties has not yet been clearly established. For example, Prives and Olden (189) found that the impairment of protein glycosylation of the acetylcholine receptor in cultured embryonic muscle cells increased the susceptibility of this receptor to degradation by cellular

proteases. In contrast, it was shown (190, 196) that glycosylation of the insulin receptor was necessary for its activation after translation, while having no effect on the susceptibility of the receptor to degradation. In the studies reported herein, it has been found, using neuraminidase treatment (Fig. 4.12), that the absence or presence of sialic acid residues on the carbohydrate chains of mature LDL receptors plays no role in determining the degradation rate of these proteins. Furthermore, the LDL receptors were not re-sialylated during the 24 hr chase period. This could suggest that recycling mostly occurs via the pre-lysosomal "short loop" route, thus bypassing the Golgi complex (the intracellular site of sialyltransferases). Alternatively, the Golgi complex may be traversed by recycling receptors in such a manner that contact with vesicles containing sialyltransferases is prevented. Resialylation of asialo-transferrin receptors in human erythroleukemia cells occurs with a half-time of 2-3 hr (221), indicating that these receptors may well recycle via the Golgi complex. There is some speculation that this route may differ from that followed by the receptor during iron uptake, since the receptor is known to recycle within minutes under such conditions (221).

#### 4.5 Concluding remarks.

In conclusion, results from these studies have shown that the half-life of LDL receptors is  $11.7 \pm 2.2$  hr in human skin fibroblasts, and this value is not unique to this cell

type. Evidence is provided for (i) the independent routing of LDL and its receptor within the cellular degradation machinery and (ii) a constant LDL receptor half-life over the range of cellular receptor protein content that was tested. Cycloheximide apparently blocks the synthesis of a rapidly turning over "factor" which is necessary for receptor degradation, and thus this agent stabilizes the protein, more-or-less doubling the half-life. In addition, the data indicate that regulation of the LDL receptor is brought about by changes in the rate of receptor synthesis only.

The present data are consistent with a pathway for LDL receptor degradation which does not necessitate procession of this protein along the coated pit-based endocytotic route, and for which the rate-limiting step is apparently not a lysosomal event.

SECTION FIVECONCLUDING DISCUSSION

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### 5.1 Introductory remarks.

Cellular receptors play a crucial role in facilitating the responses of eukaryotic cells to their environment. The actual number of receptor molecules at the surface is a key determinant of receptor function in this context. This number will be affected inter alia by dynamic processes such as the specific polypeptide synthesis, processing through the intracellular "production line", distribution to the cell surface and, ultimately, degradation. Each of these processes is complex, and is probably linked to the others to ensure optimal cell functioning. The studies described in this thesis focus on the LDL receptor system, and more particularly, on the degradation of this receptor and of its ligand (LDL), in an attempt to clarify certain mechanistic aspects of these important processes.

It is generally accepted that lysosomes are the intracellular sites for the degradation of the different structural components of LDL. Workers in this laboratory (249) have demonstrated that lysosomal cathepsins act synergistically to bring the degradation of Apo B-100 to completion. This degradation sequence occurs via a number of well-defined large intermediate fragments. In contrast, virtually nothing is known about the degradation of the receptor for this ligand. This lack of knowledge extends to very basic questions, such as the site(s) of degradation, whether the ligand (LDL) affects the rate of degradation, the role of the covalently linked carbohydrates in determining the susceptibility of receptors to degradation,

and whether receptor up- and downregulation are linked to alterations in the rates of receptor degradation.

At the start of this work, it was reasonable to assume that receptor degradation occurred in the lysosomes after frequent cycles of internalization and returning to the surface. In other cases, there are substantial data to indicate that receptor degradation is a lysosomal event. For example, the EGF receptor is degraded intralysosomally (together with its ligand) subsequent to internalization via receptor-mediated endocytosis (20). Several researchers, however, have noted that non-lysosomal mechanisms may be important in the degradation of other receptor types; this applies, for example, to insulin receptor loss in chick liver cells (135).

It should be mentioned, albeit briefly in this context, that mystery still surrounds the sorting steps resulting in the segregation of recycling receptors from other endosomal structures containing receptors (and ligands) putatively scheduled for lysosomal degradation. One possibility would be that receptors that may be destined for lysosomal degradation are extruded from the endosomal limiting membrane into the membrane of the intrusion vesicles observed in multivesicular endosomes (93). These inclusion vesicles would subsequently be combined with other luminal contents, and targeted to lysosomes. Ligands present no problem once they have been dissociated from the receptors, presumably by acidification of the environment in the endosomal compartment : the LDL molecules are presented to

lysosomes in a step(s) which ceases to operate at 20°C or lower (10).

The major focus of this thesis has been an investigation of the turnover of LDL receptors in human skin fibroblasts. The results reported in Section Four suggest that the lysosomal route is not the major initiator in this process, at least as assessed under the experimental conditions used. Leupeptin did not inhibit the degradation of the receptors (Fig. 4.10), [but did inhibit ligand degradation (Table 4.3)], indicating that one or more of the following possibilities may be operative: (i) LDL receptor degradation occurs independently of the proteinases inhibited by leupeptin; (ii) intralysosomal "downstream" steps, which do not become rate-limiting until severely inhibited, may be involved in LDL receptor degradation; or (iii) distinct lysosomal populations may exist - some for the degradation of ligand, others for that of the receptor. Alternately, entirely non-lysosomal mechanisms for receptor turnover may be operative. The observation that receptor loss still occurred at 18°C (Fig. 4.11) provides additional arguments against a classic lysosomal route, since there is a complete block in lysosomal delivery and degradation at this temperature (10).

Gevers et al (77) have proposed a general scheme for the cyclic synthesis, functioning and turnover of LDL receptors (Fig. 5.1). Based on the results reported in Section Four, various modifications to this scheme of the figure must now

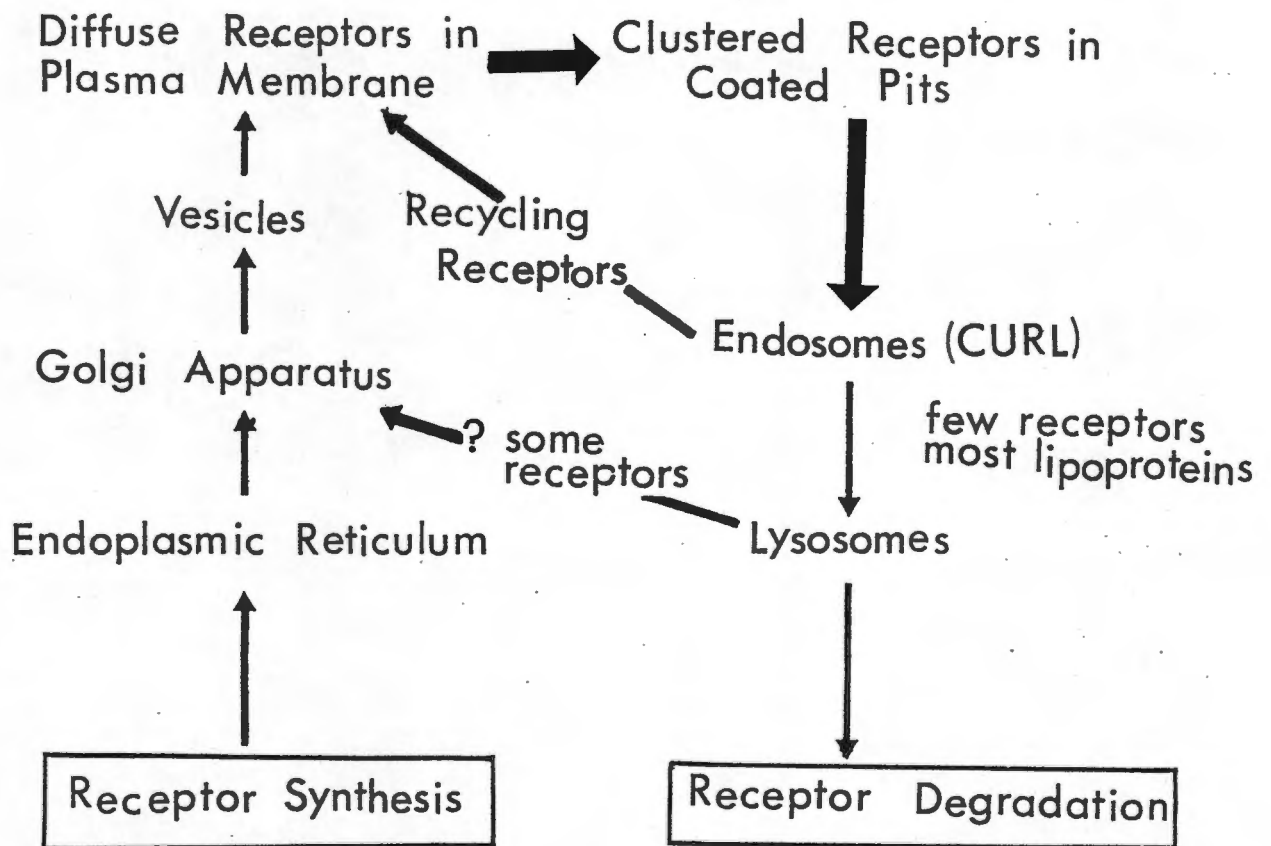


Fig. 5.1: Scheme for the cyclic synthesis, functioning and turnover of LDL receptors.

Adapted from Reference 77.

be proposed. These are explored below, and the more likely possibilities are summarized in Fig. 5.2.

## 5.2 Receptor shedding as a mechanism for cellular receptor loss.

Several investigators have documented an alternate, and somewhat novel, mechanism of transferrin receptor loss. It is well established that transferrin receptors are usually recycled subsequent to internalization (48); in reticulocytes, however, some receptors are actually lost from the cells during differentiation into erythrocytes. Such loss apparently occurs via incorporation of the transferrin receptors into the membranes of inclusion vesicles (92, 179, 180). Since these cells have few lysosomes, the multivesicular endosomes seem to escape lysosomal fusion, and lose their contents from the cell by exocytosis of multivesicular endosomes. Thus, receptor loss occurs by a process termed "receptor shedding", rather than via intracellular degradation mechanism(s).

It is tempting to speculate that cellular LDL receptor loss may be due, at least in part, to receptor shedding. This would provide a plausible explanation for the finding that participation of LDL receptors in the receptor-mediated endocytotic pathway is not an essential prerequisite for LDL receptor degradation, at rates observed in normal cells (see sections 4.3.7 and 4.4). However, experiments performed by Lehrman et al (140, 145) have excluded an LDL receptor

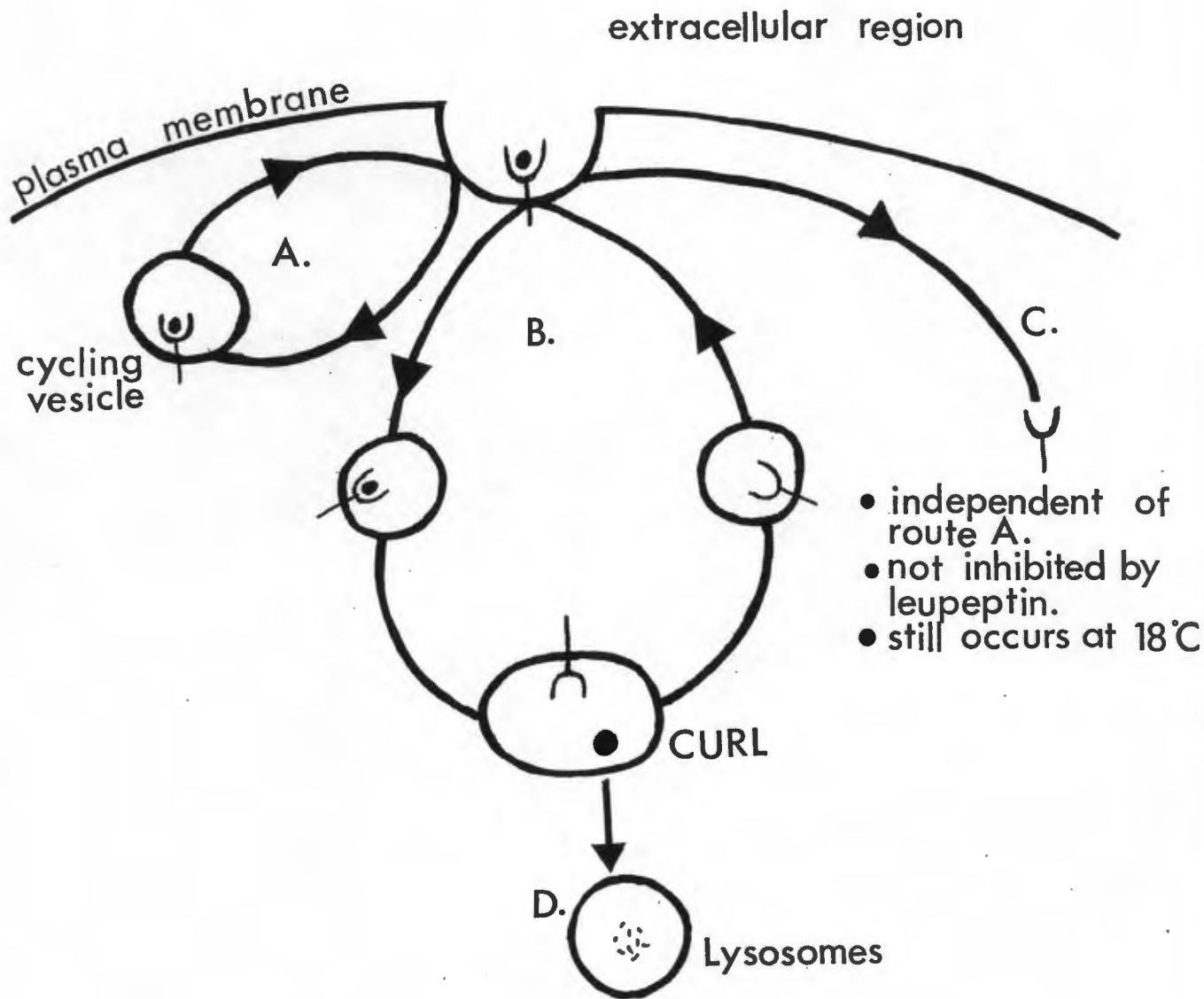


Fig. 5.2: Proposed cycling and degradation routes of LDL, and LDL receptors, to incorporate new findings obtained in this thesis.

A: Receptor cycling route ("retro-endocytosis")

B: Receptor recycling route ("receptor-mediated endocytosis")

C: Receptor degradation

D: Site of ligand degradation

Y, receptor

●, ligand

○, vesicle.

shedding mechanism. These investigators biosynthetically labeled normal fibroblasts with [ $^{35}$ S]-methionine for up to 18 hr, then subjected both the cells and the media to separate immunoprecipitations with IgG-C7. In this way, they showed that >98% of the LDL receptors were associated with the cell membrane [in contrast to the situation with receptors lacking the membrane-spanning and cytoplasmic domains (145)]. These results prove that the observed cellular loss of radiolabeled LDL receptors in normal fibroblasts must be attributable to mechanism(s) other than receptor shedding. Cellular receptor degradation presents itself as the most obvious, and likely, candidate.

### **5.3 Mechanisms of cellular LDL receptor degradation.**

The possibility that the rate-limiting steps of intracellular degradation of LDL receptors are lysosomal events, appears unlikely (section 5.1). Alternate potential mechanisms, which are non-lysosomal, are discussed below.

#### **5.3.1 Calcium-dependent neutral proteinases.**

The calcium-dependent neutral proteinases (calpains) are a novel class of thiol endopeptidases which characteristically bring about selective but limited proteolytic protein modifications (for review, see reference 187). Variations in the concentrations of proteinaceous inhibitors and activators, and in the intracellular  $\text{Ca}^{2+}$  concentrations,

apparently regulate the activity of these proteinases. Membrane-bound calpains have a low  $\text{Ca}^{2+}$  requirement (micromolar range), and are resistant to calpastatins (187); they remain sensitive to leupeptin, never-the-less, and so the inhibition data reported in section four exclude what otherwise would be a very interesting possibility.

### 5.3.2 The ubiquitin pathway.

Ubiquitin is a 76-residue protein, present in all eukaryotic cells, which participates in a complex, multi-component proteolytic pathway (for review, see reference 70), and results in the complete degradation of proteins to the amino acid level, without the involvement of lysosomes. Indiscriminate proteolysis along this pathway is prevented by a requirement for a processive ATP-dependent conjugation of target proteins with ubiquitin (42, 101). Tanaka *et al* (236) have employed a novel method to investigate the role of ubiquitin-mediated proteolysis in the accelerated degradation of HMG CoA reductase caused by the presence of 25-hydroxycholesterol. A cell line that expressed a temperature-sensitive mutation in the ubiquitin pathway was used; incubations were carried out at the non-permissive temperature, and were compared to those performed at the permissive temperature. No inhibition of the effect of 25-hydroxycholesterol on enzyme activity at the non-permissive temperature was detected. These results indicate that the increased rate of degradation of the reductase upon addition of the sterol is not dependent on the ubiquitin pathway. An

analogous approach could be used to investigate the contribution of this proteolytic system to LDL receptor degradation. It is, however, unlikely that ubiquitination is involved to any significant extent in this process, since the covalent linking of ubiquitin units to the substrate proteins is processive from an (unblocked) N-terminus (102), and in the case of mature membrane-bound receptors, this is outside the cell.

### 5.3.3 High molecular weight proteinases

Until a few years ago, little was known about cytosolic proteinases. Several, apparently different, high molecular weight proteinases (mwt range: 450 000 to 700 000 daltons) have been purified from various mammalian tissues but the relationships between these enzymes are, as yet, unclear. Some of the more recently documented high molecular weight proteinases include the "macropains" (160), the "ingensins" (112, 113, 261), and a multifunctional seryl proteinase which is present in the cytosol of all rat tissues (234). The possible contribution of such proteinases to LDL receptor degradation is an entirely speculative one at this stage.

### 5.3.4 Other possible mechanisms.

In the case of the LDL receptor, the data obtained in Section Four of this thesis clearly show that the

degradative system involved exists in "excess": irrespective of the physiological LDL receptor up- or downregulation status, the half-life remained constant, indicating that the degradation pathways are not saturated over the range of receptor amounts that were tested.

#### Denaturation.

It is not unreasonable to hypothesise that the rate-determining step in selective protein degradation may be a "physical" one - namely, the denaturation of polypeptides. LDL receptor denaturation could be followed by non-specific proteolysis at sites previously "protected" by the higher-order structure of the receptor.

#### Defective and abnormal proteins.

Defective proteins (such as those produced as a result of mutation, synthetic error or denaturation) and abnormal proteins (for example, those containing amino acid analogs), are degraded rapidly in the cytoplasm (130). On the basis of data obtained from pulse-chase studies, it would appear that some mutant forms of the LDL receptor which are synthesised in different FH patients, are turned over more rapidly than their normal counterparts (Fourie-Jacobs, Coetzee, Gevers, Van der Westhuyzen, unpublished observations). The extent to which "normal" degradative mechanisms partake in the degradation of the mutant receptors are unclear at present.

### Extracellular or cell-surface proteases.

Plasma membrane proteins offer targets for degradation by extracellular proteases and glycosidases by virtue of their externally exposed domains. The possibility therefore exists that receptor degradation is initiated by cleavages which are catalyzed by surface-bound or serum proteases. Baumann and Doyle (18) have reported the existence of a plasma membrane protein from a hepatoma cell line which is degraded by this mechanism. It is conceivable, in the case of the LDL receptor, that extracellular proteases may attack the amino terminal (outer) domain, and thereby initiate receptor degradation. In this regard, it must be noted that plasma (and extracellular fluid) contains a vast array of protease inhibitors. However, the exquisite susceptibility of LDL receptors on cells to proteases such as pronase and trypsin (84), might predispose the receptors to "extracellular" degradation.

### Intramolecular disulphide bridges.

Several cell surface receptor proteins (including the LDL receptor) are relatively abundant in cysteine residues: the role these play in the formation of intramolecular disulphide bridges and protein conformation, is a well-established one. Malbon et al (150) have recently reviewed a putative contribution of disulphide bridges to receptor activation. It appears that many cell surface receptors that are coupled to their effector systems via G proteins, possess intramolecular disulphide bridges. The cleavage of these by thiol compounds (at least in the case of the beta-adrenergic receptors) produces receptor activation similar

to that achieved by agonist binding. The relevance of intramolecular disulphide bridges to receptor protein turnover in general - and, more specifically, its possible role in LDL receptor degradation - has not been addressed yet.

#### 5.4 The proposed "N-end" rule for protein degradation.

The successful application of the postulated "N-end rule" (13), which states that the in vivo half-life of a cellular protein is a function largely of its amino-terminal residue, has so far been possible only with non-compartmentalized proteins; Bachmair et al (13) have suggested that proteins with unblocked N-termini featuring "destabilizing" amino acids lodged in the external environment may well be stable in this situation, but could be rapidly degraded if introduced into the intracellular space.

The amino-terminal sequence of the mature LDL receptor starts with three so-called "stabilizing" residues (ala, val, gly;  $t_{1/2} > 20$  hours), followed by twelve that are "destabilizing" ( $t_{1/2} < 30$  mins) - but three of these have not yet been characterized in terms of the "N-end rule" (viz. cys, asn, cys). Conceivably, a rate-limiting aminopeptidase could remove the "stabilizing" residues, thereby exposing the first "destabilizing" residue, resulting in rapid degradation of the protein. These notions all require events in which the binding domains of

the LDL receptors are exposed to the cytoplasm - no evidence for such events exists.

As a side-issue, it might be informative to consider the degradation of the LDL apolipoprotein (Apo B-100) in the context of the "N-end" rule. The amino-terminal sequence of Apo B-100 starts with three "destabilizing" residues (glu, glu, glu;  $t_{1/2}$  about 30 mins), followed by a methionine residue ( $t_{1/2}$  > 20 hours), then two "destabilizing" residues (leu,  $t_{1/2}$  about 3 mins; glu,  $t_{1/2}$  about 30 mins). When this ligand was introduced into cultured fibroblasts by scrape-loading, it was degraded with a half-life of about 50 hours, compared with that of about 1 hour subsequent to ligand uptake via receptor-mediated processes and lysosomal delivery (section 2.3.8). The former half-life is not consistent with that which is predicted according to the "N-end rule" - perhaps, after incorporation of the ligand via scrape-loading, a cytoplasmic aminopeptidase removes the three terminal glu residues, leaving exposed a "stabilizing" residue. The slow degradation of scrape-loaded LDL could reflect either (i) slow degradation by neutral (cytosolic) proteases, or (ii) slow lysosomal sequestering of the ligand. On the basis of the experiments reported in section 2.3.8, possibilities (i) and (ii) cannot be distinguished from each other. The inclusion of lysosomotropic agents in the post-load culture media, or the incubation of scrape-loaded cells at 18°C, should yield results which clarify this issue.

## 5.5 Sorting domains.

### General importance.

Domains that determine the cellular location as opposed to the function of a protein are termed "sorting domains". The steps involved in organelle-to-organelle protein sorting have been reviewed recently by Kelly (122): currently available data appear to indicate that pH sensitive receptors are involved in the unidirectional flow of proteins from one organelle to another. It is thought that newly synthesised proteins are directed to the Golgi complex; here, the proteins are sorted and routed to their correct intracellular destinations. A well-studied example of this is the mannose-6-phosphate receptor, which targets newly synthesised lysosomal enzymes from the Golgi cisternae to the lysosomes (132).

### A proposed "C-terminal" protein salvage mechanism.

It is interesting to speculate on the existence of putative sorting mechanisms for mislocated LDL receptors (a "salvage" operation). The concept of protein "salvage sequences" was formulated as a result of experimentation to investigate how proteins in the ER lumen resist export (reviewed in reference 255). Munro and Pelham (167) compared the sequences of three major ER proteins, and found that all contained the same tetrapeptide sequence (lys-asn-glu-leu) at the carboxy terminus. Removal of this sequence resulted in the secretion of the truncated proteins; the same effect was obtained by adding two extra amino acids to the tetrapeptide, but when the sequence was transferred to the

carboxy terminus of a secretory protein, the protein remained in the ER. These investigators have proposed that the tetrapeptide is an effective "salvage sequence", which could participate in the following hypothetical "salvage operation": a specific receptor (due to its location) may conceivably recognize the tetrapeptide only after the proteins (soluble in the ER lumen) have been erroneously exported from the ER. This process would require only small numbers of receptors because they would recycle between the ER and the compartment to which the proteins had been mistakenly directed. There appears, in any case, to be an intermediate compartment between the transitional elements of the ER and the Golgi stack (201) which might be rich in the salvage receptors, and which presents a possible site of protein recovery. The fundamental assumption that the tetrapeptide is available for binding is reasonable, given that the carboxy terminus should be the last part of the protein to fold, and hence should remain exposed on the protein surface.

One might consider that such a "C-terminal salvage mechanism" may play a role in the LDL receptor system. The carboxy terminal sequence of the LDL receptor is:

(C-terminus) ala-val-asp-asp-glu-leu-ser-.....(N-terminus)

The underlined tripeptide bears remarkable similarity to the tetrapeptide lys-asp-glu-leu (167). Receptors which inadvertently end up in the ER (perhaps during the complex re-routing processes involved in recycling during receptor-mediated endocytosis), could conceivably bind to the putative "salvage receptor", and hence be relocated for use.

However, if the LDL receptors remain inserted in membranes via the membrane-spanning domain, their C-terminal sequences will most likely not be exposed to vesicle lumens, and will thus not be available to bind to the "salvage receptors".

#### 5.6 A potential role for fatty acylation.

The occurrence of post-translational modification by the addition of covalently bound fatty acids to certain polypeptides has been well documented; examples are enveloped virus glycoproteins (148, 207), retroviral proteins (36, 151, 213, 217) and several eukaryotic proteins, such as the receptors for transferrin (173) and acetylcholine (171). No uniform function has yet been established for such "direct" protein acylation. One view is that such modifications may provide hydrophobic domains to the protein, and thus serve as static anchorage points to the plasma membrane (148, 207). Alternately, a role of fatty acylation in recognition mechanisms has been proposed - particularly for the myristyl moieties of transforming proteins (214). In this regard, a significant observation is that mutation of the N-terminal glycine of the tyrosine-specific protein of Rous sarcoma virus prevents both myristylation and morphological transformation, without affecting its tyrosine-kinase activity (116, 117). Thus, it appears that myristylation of the Rous sarcoma virus is important for the subcellular localization of the molecule (36). Hedo et al (97), working on cultured human lymphocytes, have recently reported that the alpha and beta subunits of

the insulin receptor (as well as the pro-receptor) incorporate [<sup>3</sup>H]-myristic and [<sup>3</sup>H]-palmitic acids covalently. Their findings indicate the existence of two different kinds of fatty acid linkage to the protein, with chemical stabilities respectively compatible with being amide and ester bonds: the alpha subunit contains only amide-linked fatty acid, while the beta subunit has both. Omary and Trowbridge (173), in studies performed using a human leukemic T-cell line, have shown that the mature form of the transferrin receptor can be labeled with [<sup>3</sup>H]-palmitate, and have provided evidence that the fatty acid is probably covalently attached to the membrane-associated portion of the molecule (172). In addition, they found that the receptor protein was degraded with a half-life of about 60 hours, whereas the palmitate moiety turned over more rapidly (60% was lost within 12 hours). The significance of these different turnover rates has not been elucidated yet.

It should be noted that a second class of acylated eukaryotic proteins exists, in which the fatty acids are components of complex glycosyl-inositol phospholipids. One function of this acylation is unequivocal and uniform: the covalently attached phosphatidyl inositol acts as a hydrophobic anchor, and is the sole means of membrane attachment (reviewed in reference 45)

To date, no investigations have been reported concerning the absence or presence of fatty acylation as a post-translational modification of the LDL receptor. If such a modification is found to occur, the possibility exists that

it may have important implications in terms of the more elusive aspects of receptor stability, life cycle or functioning. Could this putative modification provide the recognition signal(s) for the complex intracellular sorting processes involved in receptor-mediated endocytosis? Perhaps fatty acylation facilitates the lateral movement of receptors in the membranes?

#### 5.7 The relative lack of complexity of the regulation of the LDL receptor system in comparison with several other receptor systems.

The mechanisms regulating the level of cell surface LDL receptor expression are relatively "unsophisticated" in comparison with the exquisite sensitivities and complex controls inherent in other receptor systems - notably those which mediate the cellular uptake of hormones and growth factors.

LDL receptors are synthesised as single polypeptides, and, apart from cleavage of the signal sequence, do not appear to undergo any further pre-functional proteolytic processing. Downregulation of the level of cell surface LDL receptor expression appears to be due solely to a decrease in the biosynthetic rate of the receptors (sections 4.3.2 and 4.4). By contrast, insulin receptors are synthesised as single glycoprotein precursors, which are first proteolytically cleaved to yield mature receptors, each of which comprises a disulphide-linked heterodimer (2 alpha subunits - the

ligand-binding domain, and 2 beta subunits - the tyrosine kinase domain). In addition to participating in the classically described recycling pathway of receptor-mediated endocytosis, very recent experiments performed by Podlecki et al (186) on isolated hepatocytes, using biologically active, photosensitive insulin derivatives covalently attached to insulin receptors, have generated speculation as to additional receptor routing. These investigators showed that about 5% of the internalized receptor-ligand complexes were translocated to nuclei, and have proposed that transcription of insulin-dependent genes may be stimulated by the binding of insulin receptors to specific genomic targets. Whether this nuclear binding is functionally important, and whether it involves proteolytic degradation of the receptors, is presently not clear.

A further contrasting aspect of the insulin system is that insulin-induced receptor downregulation has been attributed to an accelerated insulin receptor degradation rate, while the synthetic rate remains unaltered (see section 4.4, and references 120 & 195). Fatty acylation of the insulin receptor has also been reported; the functional significance of this is, as yet, unclear (see section 5.6 and reference 97). In addition, covalent modification of insulin receptors by phosphorylation appears to play a key role in regulating the function of these receptors: auto-phosphorylation of the beta-subunit produces an enhanced tyrosine kinase activity towards exogenous substrates, and increased receptor function, whereas phosphorylation of the beta subunit on serine (and maybe threonine) residues leads

to decreased autophosphorylation and receptor tyrosine kinase activity (reviewed in reference 220).

Phosphorylation also plays an essential role in regulating the EGF receptor functioning (220). EGF stimulation induces both a rapid autophosphorylation of tyrosine residues, and the phosphorylation of thr-654. The latter effect results in receptor internalization and tyrosine kinase inhibition. Downregulation of this receptor is brought about subsequent to internalization of the receptor-ligand complex - both receptor and ligand are degraded intralysosomally. This ensures a very rapid alteration in the level of cell surface EGF receptor expression in response to changes in the external environment.

Very recently, Kishimoto et al (127) have described a soluble kinase from bovine adrenal cytosol that phosphorylates a specific serine residue (serine 833) which is located in the cytoplasmic domain of the LDL receptor, six amino acids from the carboxy terminus. This enzyme was shown to resemble casein kinase II in its catalytic properties, but to differ in the following ways: (i) its apparent mwt is about 500 000 dalton, compared with that of 130 000 for casein kinase II, (ii) its affinity for the LDL receptor (apparent  $K_M$  about 5 nM) is much greater than its affinity for casein ( $K_M$  about 10  $\mu$ M), and (iii) polylysine inhibited its activity, whereas this agent stimulates casein kinase II. The physiologic role(s) - if any - of the LDL receptor kinase is unknown.

The steps of transduction and amplification that activate cAMP-dependent kinases illustrate the extreme complexity and regulation inherent in second messenger pathways (reviewed in reference 25). In essence, external signals (first messengers) arrive at receptor molecules in the plasma membrane where they activate a closely related family of transducer molecules (which carry signals through the membrane) and amplifier enzymes (which activate internal signals carried by second messengers). The pathways in which cAMP acts as the second messenger have both stimulatory and inhibitory receptors. Signals from these receptors converge on the amplifier enzyme (adenylate cyclase), which converts adenosine triphosphate into cAMP. G proteins act as stimulatory or inhibitory transducers governing this convergence: these G proteins are activated by guanosine triphosphate (GTP), and are curtailed by GTP hydrolysis. cAMP binds to the regulatory component of its protein kinase. This liberates the catalytic component, which may phosphorylate specific proteins regulating a cellular response. The second messengers inositol triphosphate and diacylglycerol function in the inositol-lipid pathway to produce phosphorylation of distinct sets of proteins, via equally sophisticated regulatory mechanisms (reviewed in reference 25).

These somewhat brief comparisons have been used as a basis for formulating the following generalization: it would appear that receptors that bind, and in some cases mediate, the uptake of hormones and growth factors, function within a system which is "finely tuned" to changes in the

extracellular environment, and which are capable of responding rapidly, with great precision. In comparison, the LDL receptor system appears to lack this complexity, and to operate at a more "primitive" level. Conceivably, this could be due to the nature of the ligands: while it is physiologically essential that the cellular response to altered hormonal levels should be extremely rapid (hence the need for a "sophisticated" system), it is not immediately lethal if the cellular response to altered plasma cholesterol levels is somewhat slower (hence the adequacy of a more "primitive" system)..

Abundant epidemiologic evidence indicates that plasma LDL levels in Western civilized nations are generally much higher than those found in primitive societies. On the basis of the affinity of LDL receptors for LDL (assayed in cultured human cell lines), it has been proposed that the plasma level of LDL-cholesterol in Western cultures is about five times higher than that which should, in theory, be adequate to nourish dividing and steroid hormone synthesizing body cells with cholesterol (reviewed in reference 34). Also, as evidenced by the situation in non-human mammals, a relatively lower supply of cholesterol to the liver suffices for its structural role in VLDL production. That human neonates, and humans raised on low-fat diets, have substantially lower plasma LDL-cholesterol levels (relative to their adult, Western-diet fed counterparts), substantiates the notion that these reduced levels are in fact physiologic for humans.

In a situation where LDL receptors are saturated with LDL, no mechanism exists whereby the regulation of LDL receptor activity ensures "low" plasma LDL concentrations. In fact, in the case of human beings ingesting excess dietary fats and cholesterol (a typical Western diet), LDL receptor saturation and downregulation are thought to play central roles in producing the elevated plasma LDL cholesterol levels that are commonly found. The rate of LDL removal is proportional to the number of receptors which are present on the cell surfaces. Since hepatic uptake of dietary cholesterol is mediated by chylomicron remnant receptors, which are expressed independently of cholesterol accumulation (219), excess cholesterol accumulates in the liver. This, in turn, results in a reduction in the synthesis and expression of hepatic LDL receptors. Bearing in mind the key role of the liver in whole body LDL metabolism (section one), it is obvious that the combined saturation and suppression of hepatic LDL receptors may contribute significantly to elevated plasma LDL levels when there is excess cholesterol in the diet. This invokes the speculation that the LDL receptor system is one which is "designed" to retain, rather than remove, dietary cholesterol. Viewed in a historical perspective, the physiological value of this is apparent: neonates have an essential requirement for cholesterol to facilitate cell division and growth, and primitive man (presumably ingesting a "prudent" diet), would have needed to retain and mobilize all dietary cholesterol (eg., for hormone synthesis). In the latter situation, a controlled level of LDL receptor

expression to prevent "overaccumulation" of plasma LDL, would therefore appear to be unnecessary.

#### 5.8 Concluding remarks.

In ending, let us return to the beginning.

The study of the disease, Familial hypercholesterolemia, led to the discovery of LDL receptors. Subsequent investigation of the pathways by which these receptors operate facilitated the understanding of the biochemical nature of this inherited disease. Of far greater general importance, however, was the concomitant elucidation of the process of receptor-mediated endocytosis (which was "stumbled upon", so to speak, in delineating the LDL receptor pathway). This, in turn, has emphasised the critical role played by receptors in maintaining normal cellular and whole body functioning. The study of receptors, and all aspects of their life-cycles, has rapidly become a field of intense and exciting research.

And in concluding, let us project to the future.

There are many fundamental questions - particularly concerning the segregation and routing of various receptors along different pathways, such as those involving degradation - that remain unanswered. The LDL receptor system, supplemented with data available from naturally occurring and constructed genetic mutations, is a model system on which to challenge these biologically important unknowns.

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APPENDIXChemicals, Enzymes and Culture ware.

These were obtained from the following suppliers:

Corning Ltd. (Stone, England)	All plastic culture ware
Flow Laboratories (Ayrshire, Scotland)	All tissue culture media
Difco Laboratories (Detroit, MI, USA)	Trypsin (1:250), tryptose phosphate broth
Hoechst Pharmaceuticals (Johannesburg, South Africa)	Penicillin G and Streptomycin sulphate
State Vaccine Institute (Cape Town, South Africa)	Fetal calf serum
Western Province Blood Transfusion Service (Cape Town, South Africa)	Normolipidemic human blood
Aldrich Chemical Co. (Milwaukee, WI, USA)	Pristane (2,6,10,14-tetramethyl pentadecane)
BDH Chemicals Ltd. (Poole, England)	Ammonium chloride, cycloheximide, dimethylsulphoxide, hydrogen peroxide, sodium salicylate
Bio-Rad Laboratories (Richmond, CA, USA)	Bisacrylamide (NN'-methylene-bisacrylamide), TEMED (NNN'H-tetramethylene diamine)
Boehringer Mannheim (Mannheim, West Germany)	Bovine serum albumin, dithiothreitol, tris [tris(hydroxymethyl) amino methane]
Calbiochem Behring Diagnostics (San Diego, CA, USA)	Monensin, neuraminidase

Cappel Laboratories (Malvern, PA, USA)	Goat anti-mouse IgG, goat anti-rabbit IgG, rabbit anti-mouse IgG
Du Pont (NEN Res. Prods) (Hertfordshire, UK)	Cronex 4 X-ray film
Kodak (Rochester, NY, USA)	Kodak XAR 5 X-ray film
Peptide Institute, Inc. (Osaka, Japan)	Leupeptin
Pharmacia Fine Chemicals (Uppsala, Sweden)	Sephadex G-25 columns, low molecular weight marker standards
Pierce Chemical Company (Rockford, IL, USA)	Protein A-Sepharose CL-4B Iodogen (1,3,4,6-tetrachloro-3,6,-diphenylglycoluril)
Research Plus, Inc. (Bayonne & Denville, NJ, USA)	25-hydroxycholesterol
SAARChem (Muldersdrift, South Africa)	Acetic acid, chloroform, methanol, potassium bromide, sodium dodecyl sulphate, urea
Schleicher & Schuell, Inc. (Keene, N.H., USA)	Nitrocellulose BA 85 paper
Sigma Chemical Co. (St. Louis, MO, USA)	CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate, chloroquine, coomassie blue R250, glutamine, Histopaque solution, Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), Nonidet P-40, phenylalanine, sodium heparin Grade II, trypan blue dye
E. Merck A.G. (Darmstadt, West Germany)	All other reagents (Analytical Grade)

Radioactive Chemicals.

Amersham  
(Buckinghamshire, England)

[<sup>3</sup>H]-Phenylalanine  
[<sup>125</sup>I]sodium iodide

ICN Radiochemicals  
(Irvine, CA, USA)

Trans<sup>35</sup>S-label

Instrumentation.

Amicon Corp.  
(Danvers, MA, USA)

8 MC Amicon Micro-ultrafil-  
tration system

Beckman Instruments, Inc.  
(Fullerton, CA, USA)

TJ 6 centrifuge, L8-70 ultra-  
centrifuge, microfuge, Paragon  
Lipoprotein Reagent Kit,  
Beta scintillation Counter,  
Ready-Solv EP Scintillation  
fluid, DU-50 Spectrophotometer

Coulter Electronics  
(Hialeah, FL, USA)

Coulter Counter, Model Zf

Hoefer Scientific Inst.  
(San Francisco, CA, USA)

Dual Temperature Slab Gel  
Dryer Model 1150

Millipore Corp.  
(Bedford, MA, USA)

Millipore filters

Packard Instruments, Inc.  
(Downers Grove, IL, USA)

PGD-Autogamma scintillation  
counter

Pharmacia Fine Chemicals  
(Uppsala, Sweden)

FBE 3000 Electrophoretic  
System

Saint Amand Manufacturing  
Company  
(San Fernando, CA, USA)

Disposable plastic pasteur  
pipettes (Style No. 202)